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(54) Title: METHODS OF IDENTIFYING POTENTIALLY THERAPEUTICALLY EFFECTIVE AGENTS AND CELL STRAINS FOR USE THEREIN

(57) Abstract

The present invention is to a method of screening agents for potential therapeutic efficacy. The method comprises exposing a cell strain that is sensitive to known apoptotic agents to said apoptotic agents and to a potential therapeutic agent. The cell strain is then cultured and cells are removed that display diminished adherence. The remaining, adherent, cells are then incubated in the presence of a proteinase to release proteinase sensitive cells. The proteinase sensitive cells are removed to yield proteinase resistant cells and the proteinase sensitive cells are counted. The proteinase resistant cells are then collected and counted. The agent is determined to have potential therapeutic efficacy if the ratio of proteinase sensitive cells to proteinase resistant cells changes relative to a control as a result of the presence of the agent.

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METHODS OF IDENTIFYING POTENTIALLY THERAPEUTICALLY EFFECTIVE AGENTS AND CELL STRAINS FOR USE THEREIN

FIELD OF THE INVENTION

The present invention is directed to methods to detect therapeutically effective agents, particularly those that affect apoptosis, mutagenesis, cellular proliferation and cellular differentiation.

BACKGROUND OF THE INVENTION

Apoptosis is a normal physiologic process that leads to individual cell death. This process of programmed cell death is involved in a variety of normal and pathogenic biological events and can be induced by a number of unrelated stimuli. Changes in the biological regulation of apoptosis also occur during aging and are responsible for many of the conditions and diseases related to aging. Recent studies of apoptosis have implied that a common metabolic pathway leading to cell death may be initiated by a wide variety of signals, including hormones, serum growth factor deprivation, chemotherapeutic agents, and ionizing radiation. Wyllie (1980) Nature, 284:555-556; Kanter et al. (1984) Biochem. Biophys. Res. Commun., 118:392-399; Duke and Cohen (1986) Lymphokine Res., 5:289-299; Tomei et al. (1988) Biochem. Biophys. Res. Commun., 155:324-331; and Kruman et al. (1991) J. Cell. Physiol., 148:267-273.

Although agents that affect apoptosis have therapeutic utility in a wide variety of conditions, it

has not been possible to screen for these agents based on their apoptotic modulating activity. Such assays require a cell strain that can be maintained *in vitro* and retain sensitivity to apoptosis modulating signals. The vast majority of cell lines used to screen agents are selected for their ability to be maintained *in vitro*. Cells that are most easily maintained are "transformed" cells that have lost the ability to undergo apoptosis and are thus unsuitable for use in screening apoptosis modulating agents. Although cell strains such as the mouse embryonic C3H-10T½ type have been shown to be sensitive to such agents, it has not been possible to perpetuate a phenotypically stable strain of these cells beyond 120-140 population doublings. Thus, it has been impossible to obtain the reproducible results necessary to engineer a high through-put replicate culture assay for drug screening.

Most of the data about the morphology and biochemical mechanisms of apoptosis has been obtained using proliferating cells. Much less is known about death of nondividing terminally differentiated cells although the significance of its role in various types of pathological processes is well documented. Moreover, molecular mechanisms that regulate apoptosis in dividing and nondividing cells can differ widely.

In many cases depletion of growth factors induces apoptotic cell death. C3H-10T½ and Balb/c-3T3 death can be induced by serum deprivation. Tomei, In: "Apoptosis: The Molecular Basis of Cell Death" (Tomei and Cope eds.) CSHL Press, pp. 279-316 (1991); Kanter et al. (1984); and Tomei et al. (1993) Proc. Natl. Acad. Sci. USA 90:853-857. IL-2, IL-3 or IL-6 dependent lymphoid cells die in the absence of their respective growth factors. Duke and Cohen (1986) Lymphokine Res. 5:289-299; and Tai et al. (1991) Clin. Exp. Immunol. 85:312-

316. Although in some cases the anti-apoptotic effect of these factors can not be accounted for by their mitogenic effects alone, the data demonstrate that proliferation can often prevent cell death. Cold shock can induce apoptosis in cell cultures near the confluent state or growing without serum, but not if they are in the exponential phase of growth. Liepins and Younghusband (1985) Exp. Cell Res. 161:525-532; and Soloff et al. (1987) Biochem. Biophys. Res. Comm. 145:876-883. These observations do not mean that cells can not die by apoptosis if induced to proliferate. It is more likely that signals which induce apoptotic death of nonproliferating cells are ineffective in proliferating ones.

In some systems, cell proliferation is necessary for cell death. Recently it was shown that negative selection of T and B lymphocytes is a result of their apoptotic death following contact with antigen. The process can be modeled by crosslinking some surface lymphocyte antigens with antibodies. Smith et al. (1989) Nature 337:181-184; Shi et al. (1989) Nature 339:625-626; MacDonald and Lees (1990) Nature 343:642-644; Murphy et al. (1990) Science 250:1720-1723; and Motyka and Reynolds (1991) Eur. J. Immunol. 21:1951-1958. Both T and B cell lines must divide before apoptosis is initiated. Peripheral lymphocytes can be induced for apoptosis by anti-TcR only after treatments that stimulate their entrance into the cell cycle.

Many apoptosis-inducing treatments also cause blockage or a delay in cell cycle. Afanasyev et al. (1991) In: "Chemical Carcinogenesis 2, Modulating Factors" (Columbano et al. eds.) Plenum Press, New York, pp. 421-431; Kruman et al. (1991) J. Cell. Physiol. 148:267-673; Harmon et al. (1979) J. Cell. Physiol. 98:267-278; and Smets et al. (1983) J. Cell. Physiol.

116:397-403. This may seem to be important for induction of apoptotic cell death. The product of the tumor suppressor gene p53 which arrests cells with damaged DNA in G1 (Kuerbitz et al. (1992) Proc. Natl. Acad. Sci. USA 89:7491-7495) induces apoptotic death of tumor cells. Oren (1992) Cancer and Met. Rev. 11:141-148; Yonish-Rouach et al. (1993) Mol. Cell. Biol. 13:1415-1423; and Ryan et al. (1993) Mol. Cell. Biol. 13:711-719. Blockage of the cell cycle is absolutely necessary for subsequent apoptosis in this system. Activation of c-*myc*, a gene normally involved in regulation of cellular proliferation, induces apoptosis in cells arrested in cell cycle. Evan et al. (1992) Cell 69:119-128; Bissonnette et al. (1992) Nature 359:552-556; and Shi et al. (1992) Science 257:212-214.

Protein synthesis and gene activity play a distinctive role in apoptosis. In many instances DNA degradation and apoptotic cell death can be prevented by inhibitors of RNA or protein synthesis. Umansky (1982) J. Theor. Biol. 97:591-602; Beaulaton and Lockshin (1982) Int. Rev. Cytol. 29:215-235; Smith et al. (1989); Martin and Johnson (1991) In: "Apoptosis: The Molecular Basis of Cell Death" (Tomei and Cope, eds.) CSHL Press, pp. 247-261; and Umansky, ibid., pp. 193-208. The appearance of new proteins has been found to precede DNA cleavage in different model systems. Buttyan, ibid., pp. 157-173; Wadewitz and Lockshin (1988) FEBS Lett. 241:19-23; Domashenko et al. (1990) Int. J. Radiat. Biol. 57:315-329; and Lockshin and Zakeri (1991) In: "Apoptosis, The Molecular Basis of Cell Death" (Tomei and Cope, eds.) CSHL Press, pp. 47-60. However, in other experimental systems (e.g., cell death induced by cytotoxic lymphocytes or natural killer cells), apoptosis is not inhibited by agents that inhibit protein synthesis. Duke, ibid., pp. 209-226. TNF-induced apoptosis was even

facilitated by inhibitors of protein and RNA synthesis. Rubin et al. (1988) Cancer Res. 48:6006-6010. Some established cell lines that are normally insensitive to TNF lose their resistance in the presence of cycloheximide. Moreover, both cycloheximide and actinomycin can induce apoptotic death of some cells. Tomei (1991); and Collins et al. (1991) Br. J. Cancer Res. 3:518-522. It is likely that there are two classes of proteins related to apoptosis in a cell: the first group is required for apoptosis to proceed, whereas, the second class are antagonists of the process; the prevalence of one system over another determines the final effect, namely, cell death or survival.

Recent data on gene expression support the concept that apoptosis is controlled by both positive and negative pathways. Evidence for induction of genes such as TRPM-2, transglutaminase, RP-2 and RP-8 and several protooncogenes in different apoptotic systems is consistent with the view that apoptosis is modulated although there is no clear evidence of their role in actively mediating apoptotic DNA cleavage. These genes are described, inter alia, in: Buttyan (1991); Lockshin and Zakeri (1991); Fesus et al. (1987) FEBS Lett. 224:104-108; Owens et al. (1991) Mol. Cell. Biol. 11:4177-4188; Buttyan et al. (1988); and Kyprianou and Isaacs (1989) Mol. Endocrinol. 3:1515-1522. It has been shown that the product of the p53 anti-oncogene can induce the apoptotic death of transformed or tumor cells. Yonish-Rouach et al. (1991) Nature 352:345-347. Conversely, the activity of the bcl-2 gene has been associated with suppression of apoptosis in some lymphoid and other cell lines. Vaux et al. (1988) Nature 335:440-442; Hockenbery et al. (1990) Nature 348:334-336; and Korsmeyer (1992) Blood 80:879-886. An analogous gene with similar activity (ced-9) was found in *C. elegans*.

Hengartner et al. (1992) Nature 356:494-496. It is significant that some cells which express the TNF receptor are insensitive to TNF, although they possess the same amount of receptors as sensitive cell lines. Paul and Ruddle (1988) Ann. Rev. Immunol. 6:407-438; and Tsujimoto et al. (1985) Proc. Natl. Acad. Sci. USA 82:7626-7630. This suggests that factors that promote apoptosis also induce a reactive inhibiting system that may act either intracellularly or intercellularly. It is noteworthy that the *bcl-2* protein is found only in cells surviving in tissues characterized by apoptotic cell death. Hockenbery et al. (1991) Proc. Natl Acad. Sci. USA 88:6961-6965.

Thus, it is evident that changes of gene activity, both activation and/or repression, are an obligatory step for most cases of apoptotic cell death.

It has now been found that, by the method described herein, cell strains sensitive to apoptotic agents can be used to provide reproducible results in screening for agents that modulate a wide variety of disorders. These phenotypically stable cell strains can now be maintained for at least 900 population doublings. Moreover, by utilizing the parameters of the screening assay, new, improved cell strains can now be obtained that are suitable for use in the screening assays and for studying apoptosis.

SUMMARY OF THE INVENTION

The present invention is directed to a method of screening agents for potential therapeutic efficacy. The method comprises culturing a cell strain that is sensitive to apoptotic modulating agents for a period of time and under conditions sufficient to attain

exponential proliferation activity without density-dependent constraints at any time during the assay. The cell strain is then exposed to conditions that are known to induce apoptosis and either simultaneously or shortly thereafter exposed to an agent to be screened. The cell strain is cultured for a time and under conditions suitable to attain a density of about one population doubling from density-dependent arrest of cell cycle. Cells that display diminished adherence are then removed and enumerated. The remaining adherent cells are then incubated in the presence of a proteinase for a time and under conditions suitable to release proteinase sensitive cells from the adhesion surface. The proteinase sensitive cells are removed and enumerated to yield the remaining proteinase resistant adherent cells. The proteinase resistant cells are then incubated for a time and under conditions suitable to release the cells. The released cells are collected and enumerated.

It is possible that treatment of certain diseases requires that therapeutic agents either specifically induce apoptosis in instances where deletion of cells is believed to be therapeutically effective such as in the treatment of cancer. Inhibition of apoptosis is preferred in instances where prevention of cell loss is believed to be therapeutically effective such as in prevention of ionizing radiation-induced cell death in the gastrointestinal system. The agent is determined to have potential anti-apoptotic therapeutic efficacy if the number of non-adherent cells is reduced relative to the number of non-adherent cells in the control, and the number of proteinase resistant cells increases relative to a control. The agent is determined to have apoptosis inducing therapeutic efficacy if there is an increase in the number of non-adherent cells relative to the number of non-adherent cells in the control.

The method can also be used to obtain and maintain phenotypically stable cell strains suitable for use in the screening assay or any other assay requiring cells that are responsive to apoptosis inducing and apoptosis inhibiting agents.

The method can also be used to screen agents for potential apoptosis modulating activity in terminally differentiated cells. The method is identical to that described above except that the cells are allowed to become quiescent prior to adding the agent to be screened. Additionally, the agents can be screened in the presence or absence of serum. Preferably, the assays are performed in the absence of serum as the cells are more sensitive to the agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow chart of the cell culture method described in Example 1.

Figure 2 is a bar graph depicting apoptotic/trypsin sensitive/trypsin resistant cell count analysis at 24 hours post serum-deprivation. Figure 2 is discussed in Example 3.

Figure 3 is a bar graph depicting apoptotic/trypsin sensitive/trypsin resistant cell count analysis at 48 hours post serum-deprivation. Figure 3 is discussed in Example 3.

Figure 4 is a bar graph depicting apoptotic/trypsin sensitive/trypsin resistant cell count analysis at 24 hours post serum-deprivation of SF90, alanate 166, and alanate 180. Figure 4 is discussed in Example 3.

Figure 5a is a graph depicting the kinetics of apoptosis induced in exponential (logarithmic) phase C3H-10T½ cells by serum free media. The open circles

represent the number of dead cells and the closed circles represent the number of total cells. Figure 5b is a graph depicting the kinetics of death in quiescent C3H-10T½ cells upon induction by cycloheximide. Figures 5a and b are discussed in Example 5.

Figure 6 is a graph depicting the death of exponentially growing C3H-10T½ cells induced by serum deprivation in the presence or absence of cycloheximide. The open squares represent cells in serum; diamonds represent cells in serum and cycloheximide; filled squares represent cells in the absence of serum; and open circles represent cells in the absence of serum and the presence of cycloheximide.

Figure 7 is a graph depicting the sensitivity of C3H-10T½ to cycloheximide and serum withdrawal over the course of ten days as the cells go from exponential growth to quiescence.

Figure 8 is a bar graph depicting the percentage of dead cells upon exposure of C3H-10T½ quiescent cells to various chemicals. CH stands for cycloheximide; AM D stands for actinomycin; PURO stands for puromycin; and α-AMA stands for α-amanitin.

Figure 9 is a bar graph depicting the kinetics of cycloheximide-induced death of cardiomyocytes in the presence (shaded bars) or absence (solid bars) of serum at 24 and 36 hours.

Figure 10 is a graph depicting the dose response of cycloheximide-induced death of cardiomyocytes in the presence (filled circles) or absence (open circles) of serum at 24 hours.

DETAILED DESCRIPTION OF THE INVENTION

1. Screening Assay

The present invention is to a method of screening agents for potential therapeutic efficacy. The method comprises providing a cell strain that is sensitive to apoptotic agents; culturing the cell strain for a time and under conditions sufficient to attain exponential proliferation without density dependent constraints at any time during the assay; exposing the cell strain to conditions that are known to induce apoptosis; exposing the cell strain to an agent to be screened; culturing the cell strain for a time and under conditions suitable to attain a density of about one population doubling from density-dependent arrest of cell cycle; and removing and enumerating cells that display diminished adherence. The remaining adherent cells are then incubated in the presence of a proteinase for a time and under conditions suitable to release proteinase sensitive cells from the adhesion surface. The proteinase sensitive cells are removed and enumerated to yield the remaining proteinase resistant adherent cells. The proteinase resistant cells are then incubated for a time and under conditions suitable to release the cells. The released cells are collected and enumerated.

The agent is determined to have potential therapeutic efficacy if the number of non-adherent cells is reduced relative to the number of non-adherent cells in the control, and the number of proteinase resistant cells increases relative to a control.

The cell strain is obtained from pluripotent embryonic cells with stable, normal, intact, phenotypes. The cell strain is an adherent strain, its *in vitro* growth is dependent on anchorage to the solid support on which it is grown.

Preferably, the cell strain has the following characteristics: density dependent regulation of proliferation and death; pluripotent and capable of demonstrating at least two distinct differentiated cell types upon suitable stimulus; sensitive to loss due to transformation induced by carcinogenic and oncogenic agents such as DNA damaging agents or various oncogenic viruses; response to agents that both induce and block proliferation, differentiation, and apoptosis; ability to exhibit apoptotic cell death as marked by ultrastructural changes of chromatin and specific changes in the molecular structure of DNA associated with apoptotic DNA degradation. More preferably, the cell strain is the mouse embryonic C3H-10T½ clone 8. This cell strain is available from the American Type Culture Collection under accession number ATCC CCL 226 and is designated C3H-10T½, clone 8. C3H-10T½ has also been described for use in transformation experiments. Yavelow et al. (1985) Proc. Natl. Acad. Sci. USA, 82:5395-5399.

The initial culturing step is to attain stable exponential proliferation without density dependent constraints at any time during the assay. In the case of C3H-10T½, this is for about 5 days at 37°C with a gas overlay of 5% CO_2 in a suitable growth medium. Although the optimal medium will vary depending on the cell strain used, in the case of C3H-10T½, the preferred medium is Eagle's basal medium with Hank's salts (BME) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). In the case of C3H-T10½, after five days of incubation, the cells have reached about seventy-five percent confluency. Although this is the preferred confluency, particularly for C3H-T10½, the confluency can be greater or lesser so long as statistically significant results are obtained. Although the optimal confluency for each cell strain is derived empirically, this is a

straightforward determination based on the parameters discussed below.

Incubating the cells so that they maintain exponential proliferation without density dependent constraints during the assay prevents artificial skewing of the results. This is because once the cells reach a certain density, exponential proliferation ceases and the cells become quiescent. The density at which quiescence occurs is unique to each cell strain and is empirically derived by measuring indicia of cell division such as manufacture of DNA. Quiescent cells do not respond to apoptotic and anti-apoptotic agents in the same manner as exponentially growing cells. As discussed below, however, the screening assay may be performed using quiescent cells in order to determine the possible effect of the agents screened on terminally differentiated cells. Preferably, agents are screened separately against both exponentially growing and quiescent cells. The discussion below relates to exponentially growing cells. Quiescent cells should attain the quiescent state prior to adding the agent; however, the density dependent constraints are not necessary. Preferably, the cells are exposed to the agent shortly after entering quiescence. The cells can be tested before they undergo spontaneous apoptosis or other changes.

In the case of exponentially growing cells, the level of confluency reached before adding the apoptotic agent depends only on maintenance of the exponential growth phase and attaining a statistically significant number of cells. In the case of C3H-T10½, seventy-five percent confluency is preferred. The optimal confluency for any strain is empirically derived.

The condition that induces apoptosis in the cells after the initial incubation step includes, but is not limited to, serum deprivation, growth factor

deprivation, ultraviolet radiation, γ-radiation, soft β-radiation, hypo-osmotic shock, chemotherapeutic agents, or specific receptor mediated agents. In the case of C3H-10T½, the preferred method of inducing apoptosis in a reproducible portion of the cell strain in replicate cultures is to replace the serum-supplemented growth medium with serum-free growth medium. After the change to serum-free medium, the cells are incubated for a time and under conditions suitable to maintain exponential proliferation without density dependent constraints in suitable replicate control cultures. In the case of C3H-10T½, the conditions are for about 20 hours at 37°C in an atmosphere of 5% CO_2.

Culturing of cells is dependent upon FBS supplement to the synthetic growth medium. It is important to preserve both the growth promoting potential of serum supplements as well as the sensitivity to apoptosis inducers. It has now been found that serum production lots vary not only with respect to proliferative potential, but also with respect to the ability of the serum to promote apoptosis responsiveness in the cells. This aspect of the assay is counter-intuitive in as much as conventional serum screening tests used in the art are based primarily upon determining the relative ability of various production batches of animal sera to maintain high viability or survival rates of various reference cell cultures. Contrary to that rationale, the apoptosis assay serum screening test determines the relative ability of various serum production batches to yield apoptotic, or dying and dead cells upon withdrawal of the growth medium. Thus, each serum lot must be selected from a screened group of several lots in order to ensure preservation of the cell strain responsiveness to both apoptosis inducers and inhibitors.

The agents to be assayed are introduced to the cells either simultaneously with or shortly after induction of apoptosis. Although the time of introduction will vary according to the cell strain used and the method of induction of apoptosis, the optimal parameters can be determined empirically and applied in a routine basis thereafter. In the case of C3H-10T½, the agent to be assayed is added simultaneously with induction of apoptosis.

After addition of the putative therapeutic agent, the cells are further incubated for a time sufficient to display apoptosis. At that time, a certain proportion of the cells in each replicate culture display rounding and loss of flattened shape, diminished adherence and are removed by a mild shearing effected by washing with a suitable buffered salt solution.

After the nonadherent cells are removed and enumerated, the remaining adherent cells are comprised of proteinase sensitive and proteinase resistant cells. It has now been found that the proteinase sensitive cells are the population that contains the cells that would have otherwise died by apoptosis in the absence of an effective apoptosis inhibitor. The proteinase sensitive cells are removed for enumeration by incubation with a suitable proteinase. A suitable proteinase is trypsin. Trypsin is the standard proteinase for removing adherent cells; however, any proteinase that effectively removes proteinase sensitive cells without damaging the cells removed or the remaining proteinase resistant cells is suitable for use in the present invention.

In the case of C3H-10T½, the adherent cells are incubated in the presence of concentrations of about 1 mg/ml to 1 μg/ml trypsin for about 10 minutes with gentle shaking at room temperature and are removed by washing.

The proteinase sensitive cells are then enumerated by a suitable method.

Suitable enumeration methods include, but are not limited to, direct physical counting, electronic particle counting and fluorescence staining to measure DNA. Any cell counting means known in the art is suitable for use in the present invention.

The proteinase resistant cells are then released. Preferably, release is accomplished by incubation in the presence of an agent that decreases the calcium concentration to a level sufficient to release the cells. Such agents include, but are not limited to, calcium chelators such as ethylenediaminetetraacetic acid and ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetraacetic acid.

The number of agents suitable for assaying for therapeutic effectiveness is virtually limitless and can be obtained from a variety of chemical, nutritional and biological sources. For instance, suitable chemical agents can be novel, untested chemicals, as well as agonists, antagonists, or modifications of known therapeutic agents. Nutritional agents can be complex extracts from plant or animal sources or extracts thereof. Such agents can be easily derived from plant sources such as soy, pea, or potato by-products, or from animal products such as whey or other milk byproducts. Biological agents include biological response modifiers, antibodies and other small molecules. Such small molecules can be either derived from biological sources or chemically synthesized to mimic the effect of biological response modifiers.

The assay is suitable for use in a high throughput format, thus enabling the screening of large numbers of agents in a range of concentrations. Most therapeutic agents screened to date have been effective

in a concentration of about 1 μg/ml, although a wide range of concentrations can be screened. Preferably, the range of concentrations is from 1 ng/ml to 1 mg/ml. The upper range is limited by solubility of the agent and the lower range is not necessarily limited but should be in a therapeutically effective range.

The assay is preferably performed in standard tissue culture 60 mm Petri dishes which have about 20 cm^2 surface area. Although the assay may be scaled down to 2 cm^2 tissue culture dishes, it is not suitable for use in microtiter plates having significantly lower growth surface areas. The cell strain must be maintained as a spatially random distributed population that can not be maintained in the small surface area of a microtiter dish.

A positive response to the agents is clear. Typically the agents found to have therapeutic efficacy by decreasing apoptosis have been found to increase proteinase resistant cells in a statistically significant manner. These agents also statistically reduce the number of non-adherent, apoptotic cells.

It has also now been shown that agents that reduce the number of non-adherent cells may do so through a non-specific action unrelated to reduction of apoptosis. Thus, merely measuring the number of non-adherent cells that appear in replicate treated cultures is subject to error in the form of false positive results and is insufficient for the screening assay. For instance, toxic agents that may rapidly damage cells and lead to disintegration of the cells may be falsely determined to reduce the appearance of apoptotic cells by virtue of the reduction in non-adherent cell counts. This has now been found to be an avoidable error if proteinase resistant cells are simultaneously determined in the replicate cultures. Also, enumeration of a

proteinase resistant cell number is inherently more reliable than measurement of a loss of cells by a subtraction method.

The cell strain utilized in the assay is particularly sensitive to apoptosis inducers and inhibitors and is therefore useful in identifying agents with therapeutic efficacy in treating diseases and conditions related to aging and cellular differentiation. These conditions and diseases include but are not limited to cardiovascular disease, cancer, immunoregulation, viral diseases, anemia, neurological disorders, diarrhea and dysentery, diabetes, hair loss, rejection of allografts, prostrate hypertrophy, obesity, ocular disorders, stress, immunodeficiency or immunosuppression including AIDS, and the aging process itself.

The assay is not limited to diseases and conditions related to aging, however, as the list above indicates, many conditions associated with aging may also be the result of illness or other physical insults. For instance, while not related to aging per se, the trauma induced by head and spinal cord injuries results in apoptotic events that cause the subsequent cellular loss and the consequent exacerbation of paralysis in many patients. Agents useful in preventing apoptosis will be useful in minimizing such paralysis.

It has now been found that the assay further distinguishes between agents that have therapeutic utility and those that block apoptosis but are toxic. By comparing the proportion of apoptotic, proteinase sensitive and proteinase resistant cells, the assay detects those agents that modulate proteinase sensitivity (the apoptosis marker) rather than those agents that merely decrease the number of nonadherent (dead) cells.

The assay further provides the means to determine effects of agents on proliferation such as

those which have mitogenic activity in addition to anti-apoptotic activity.

The data presented in the following examples indicate that there is a differential between the sensitivity of exponentially growing and quiescent C3H-10T½ cells to apoptosis-inducing treatments. Exponentially growing cells are extremely sensitive to serum deprivation, resistant to inhibitors of RNA or protein synthesis, but quiescent C3H-10T½ cells are resistant to serum deprivation and rapidly die upon inhibition of protein or RNA synthesis. The results also indicate that the sensitivity of exponentially growing C3H-10T½ cells can be correlated to dividing cells *in vivo*; whereas the sensitivity of quiescent C3H-10T½ cells can be correlated to terminally differentiated cells *in vivo*. The assay further provides a method of detecting the effect of apoptosis modulating agents or potential apoptosis modulating agents on cells in different stages of the cell cycle.

2. Method of Obtaining Suitable Cell Strains for the Screening Assay

The invention further provides a method of obtaining cell strains other than C3H-10T½ that retain responsiveness to apoptosis modulating agents. The method, similar to that above, comprises obtaining a cell sample; culturing the cell sample for a time and under conditions sufficient to maintain exponential proliferation without density dependent constraints; exposing the cell sample to conditions that induce apoptosis; treating the cell sample with an agent known to induce proteinase resistance; culturing the cell sample for a time and under conditions suitable to attain a density of about one population doubling from density-dependent arrest of cell cycle; removing cells that

display diminished adherence to yield adherent cells; exposing the adherent cells to a proteinase; incubating the adherent cells for a time and under conditions suitable to release proteinase sensitive cells; and collecting the remaining proteinase resistant cells. These proteinase resistant cells are then retained and passaged to generate a stable cell strain that continues to express responsiveness to apoptosis inducers and inhibitors.

The preferred treatment of cells for maintenance of apoptosis responsiveness is 12-O-tetradecanoyl phorbol-13-acetate (TPA) at a concentration of about 10^{-9} to 10^{-8} molar and is commercially available from Sigma Chemical Company and others.

The incubation conditions and other parameters are essentially similar to those described for the assay described above.

The cell sample is preferably obtained from pluripotent embryonic cells with a normal, intact, phenotype. The species the cell sample is derived from is not as important as the embryonic origin of the cells. This is because embryonic cells are controlled by highly conserved, common proteins that exhibit minimal species specific variations important to adult organisms. The cell strain should not be transformed, that is, should not be a so-called "immortalized" or tumorigenic cell line. Such immortal cell lines have lost most or all of their ability to respond to apoptosis modulating signals or agents.

3. Method of Passaging the Cell Strain

The invention further provides a method of passaging the cell strains so as to preserve phenotypic fidelity defined in part but not completely by cellular responsiveness to apoptosis modulating agents. The

method comprises culturing a cell strain that is sensitive to apoptotic modulating agents for a time and under conditions sufficient to attain exponential proliferation activity without density-dependent constraints at any time during the method. The cell strain is then exposed to conditions that are known to induce apoptosis. The cell strain is cultured for a time and under conditions suitable to attain a density of about one population doubling from density-dependent arrest of cell cycle. Cells are then removed that display diminished adherence and enumerated. The remaining adherent cells are then incubated in the presence of a proteinase for a time and under conditions suitable to release proteinase sensitive cells from the adhesion surface. The proteinase sensitive cells are removed and enumerated to yield the remaining proteinase resistant adherent cells. The proteinase resistant cells are then incubated for a time and under conditions suitable to release the cells. The released cells are collected and enumerated.

The incubation conditions and other parameters are essentially similar as those described for the assay described above.

The following examples are provided to illustrate but not limit the claimed invention. The examples describe the identification of therapeutically effective agents by the use of the present invention.

Examples

Example 1
Cell Culture Techniques

Figure 1 depicts a flow chart of the basic technique of cell culture for the assay. The following is a more detailed description of the assay as performed using C3H-10T½ clone 8 mouse embryonic cells.

The cells are obtained at the lowest available serial passage level preferably less than level 11. The phenotypic characteristics of the cells are verified as meeting the following criteria. Standard cell culture techniques are used. The steps presented below are those used with exponentially growing cells. In the case of quiescent cells, the cells are allowed to reach quiescence and the density-dependent constrains need not be observed. Moreover, the assays utilizing quiescent cells can be performed in the presence or absence of serum although they are more sensitive in the absence of serum. The use of quiescent cells is described in Examples 5 and 6.

1. Confirmation of a mean doubling time of 22 ($\pm$2) hours under standard conditions of basal Eagle's growth medium supplemented with 10% (v/v) FBS in plastic culture flasks maintained at 37° C in a humidified atmosphere of 5% CO_2.

2. Cloning efficiency determined to be 25% ($\pm$2) at densities of 200 cells/20 cm^2 under standard growth conditions.

3. Saturation cell density confirmed to be 5 x 10^5 ($\pm$2 x 10^5) cells/20 cm^2 plastic petri dish under standard growth conditions.

4. At saturation cell density, it is confirmed that more than 98% of cells are in the G_1 phase of the cell cycle.

5. The morphology of exponentially proliferating cultures is radically changed at saturation density such that the spindle shaped cells having extensive overlapping and lack of parallel orientation during exponential proliferation changes to wide, flat epithelioid monolayer without distinct intercellular demarcation and no overlapping.

6. Cells are sensitive to malignant transformation by chemical carcinogens, typically 3-methyl cholanthrene, or ultraviolet irradiation yielding transformed foci.

7. Cells do not form a fibrosarcoma tumor when injected at levels of 10^5 cells subcutaneously in the suprascapular region of syngeneic animals, whereas, following malignant transformation *in vitro*, tumors are observed under similar conditions.

The cells are then cultured in containers that are typically 60 mm diameter plastic petri dishes specially prepared for mammalian cell culture and are commonly available from several commercial sources.

Cells for replicate culture are obtained from stock cultures which are confirmed to be in exponential phase proliferation and not in post-confluent saturation density to ensure that cells are not arrested in the G_1 phase of the cell cycle.

The cells are seeded onto each plate in a volume of 5 ml complete growth medium in which are suspended a standardized number of cells. The standardized number should be not less than 10^3 but not greater than 10^4 cells.

Special care must be taken to ensure that cells are uniformly distributed over the surface of each

culture dish in order to prevent clustering of cells during subsequent growth which results in non-uniform increases in cell densities and premature saturation densities in limited regions on each dish. Premature saturation density in limited regions results in serious errors in assay results.

Growth medium is renewed each 48 hours.

When cell density reaches approximately 70% uniformly across each dish surface, which typically corresponds to a density of 1×10^5 to 3×10^5 cells/dish, the complete growth medium is removed by aspiration and replaced with fresh serum-free growth medium.

Drugs or agents to be assayed can be premixed into the serum-free medium, or added in appropriately small volumes immediately after the medium change.

Typically, in order to ensure statistical reliability, each agent or specific treatment is performed on at least four replicate cultures and appropriate controls are also incorporated.

After a standardized period of incubation at 37°C under humidified 5% CO_2 atmosphere of between 3 hours and 72 hours, typically 24 hours, each plate is prepared for measurement of responses.

The following measurements are performed:

1. All non-adherent or loosely adherent cells are removed from the culture dish and measured by appropriate techniques typically counting by electric particle counting instrument.

2. The remaining adherent cells are exposed to a buffered (typically pH 7.3) balanced salt solution such as Hanks Balanced Salt Solution containing a standardized concentration of the enzyme trypsin. The trypsin concentration is typically 0.1 mg/ml but can be between 1 and 0.001 mg/ml, typically in a volume of 1 ml.

Each culture is incubated either at ambient temperature or 37°C on a rocking platform to ensure uniform distribution of the trypsin reagent over the culture surface. After a standardized period of typically 10 minutes, the released cells are removed from each dish, and measured by the same means described above, typically electronic particle counting. This measurement is referred to as the serum deprivation released or SDR count and typically contains at least 98% apoptotic cells.

The remaining adherent cells in each dish are then released by exposure to a buffered solution containing a calcium ion chelating agent typically EDTA typically at a concentration of 2 mg/ml. This measurement is referred to as the proteinase sensitive or PS count and typically contains the cells that would have otherwise died by apoptosis in the absence of an effective inhibitor.

The final cells remaining adhered to the solid support are then immediately dispersed and removed from the dish for measurement by the same means used in previous measurements, typically electronic particle counting. This measurement is referred to as the proteinase resistant or PR count and typically contains cells that express the property of resistance to proteinased-induced shape change which has been identified to be a critical specific expression related to control of apoptosis.

Each cell count is typically performed in duplicate on each of four replicate dishes for each experimental treatment group and control. The statistical significance of this is shown in Example 4.

Final data are initially expressed as the mean cell number and standard deviation of the mean according to conventional statistical data-analysis as discussed in

Example 4. The results obtained are shown in Table 1. The numbers in Table 1 printed in bold print represent the numbers used in Table 2 as described in Example 2. In Table 1, the data are reported as cells/plate x 10,000. The abbreviations used are: SD, standard deviation; PR, proteinase resistant cells; and PS, proteinase sensitive.

Table 1: Bovine serum screening test
C3H-10T1/2 cells

	Apoptotic	+/- SD	% control	PR	+/- SD	% control	PS	+/- SD	% control	Total	% control
Lot #1 Control	22.6	2.6	100.0	17.7	3.6	100.0	20.4	3.3	100.0	60.7	100.0
1 nM TPA	19.8	0.6	87.7	27.8	4.4	157.1	16.7	2.6	81.9	64.3	105.9
10 nM TPA	13.1	1.5	**58.0**	48.1	4.4	**271.8**	15.9	1.2	77.9	77.1	127.0
Lot #2 Control	13.6	0.5	100.0	17.2	2.1	100.0	20.2	1.8	100.0	51.0	100.0
1 nM TPA	10.4	1.6	76.5	23.5	3.3	136.6	15.0	1.8	74.3	48.9	95.9
10 nM TPA	6.0	0.4	**44.1**	35.6	2.7	**207.0**	14.2	1.3	70.3	55.8	109.4
Lot #3 Control	30.8	2.0	100.0	16.7	5.1	100.0	25.9	5.2	100.0	73.4	100.0
1 nM TPA	25.4	1.3	82.5	26.3	4.5	157.5	22.3	2.8	86.1	74.0	100.8
10 nM TPA	15.4	1.5	**50.0**	49.5	1.8	**296.4**	13.3	1.2	51.4	78.1	106.4
Lot #4 Control	35.9	1.9	100.0	20.5	1.0	100.0	34.6	0.9	100.0	90.9	100.0
10 nM TPA	21.6	4.9	**60.2**	42.6	6.5	**207.8**	45.0	3.4	130.1	110.1	121.1
50 nM TPA	16.8	1.4	46.8	47.4	4.2	231.2	45.9	4.1	132.7	109.1	120.0
Lot #5 Control	49.1	6.0	100.0	20.5	4.2	100.0	49.6	2.5	100.0	119.2	100.0
10 nM TPA	30.7	5.1	**62.5**	53.9	2.5	**262.9**	47.0	4.9	94.8	131.6	110.4
50 nM TPA	16.7	2.4	34.0	55.6	6.7	271.2	52.2	3.2	105.2	124.5	104.3

Initial data-analyses for assay reliability should reveal that variations among replicate culture dishes for any of the three measurements should not be greater than 7% standard deviation. Data can be normalized by expressing the individual counts of SDR, PS, and PR as ratios to the corresponding value determined in control dishes.

A preferred positive result is typically dependent upon a statistically significant reduction of SDR cells in combination with a statistically significant increase in PR cells. However, putative apoptosis modulating agents can produce either reduction of SDR cells or increased PR cells and should be considered to be positive and warranting further consideration. Agents that produce either increased SDR or decreased total cell counts (i.e., SDR + PS + PR) should be considered to be potentially cytotoxic at the concentrations applied. A negative outcome would be failure to observe changes in either SDR or PR counts at concentrations determined to be non-toxic.

Example 2

Bovine Serum Screening Test

The purpose of screening the bovine serum used as a supplement to the synthetic portion of the cell culture growth medium is to determine the best manufacturer's production batch in terms of optimal assay performance. This aspect of the assay is counter-intuitive in as much as conventional serum screening tests used by those skilled in the art are based primarily on determining the relative ability of various production batches of animal sera to maintain high viability or survival rates of various reference cell cultures. Contrary to that rationale, the apoptosis assay serum screening test determines the relative

ability of various serum production batches to yield apoptotic, or dying and dead, cells upon withdrawal from the growth medium.

As an example, Tables 1 and 2 present typical data obtained in the process of screening 5 different bovine serum production batches obtained from a commercial source. The cells were screened as described in Example 1 with exponentially growing cells. These are listed as Lots 1 through 5. In order to distinguish between necrotic and apoptotic cells, cells are treated with TPA. Two independent variables to be measured are the number of apoptotic cells in the untreated control cultures compared with replicate cultures treated with TPA. These variables are the released cell or Apoptotic count, and the proteinase-resistant or PR cell count as shown in Table 1.

Results are evaluated as follows: Apoptotic and PR responses are expressed in terms of a ratio or percentage of the corresponding response obtained in untreated replicate cultures. A desirable response would be simultaneous maximal reduction of Apoptotic cell count and enhancement of PR cell count. Therefore, the responses are then ranked according to each lot's respective response for each variable as shown in Table 2.

Table 2: Analysis of results of screening data
Activity determined at 10 nM TPA

	serum lot #	rank	Suppression of apoptosis	rank	Induction of PR	total ranking
	1	3	58.0	2	272	5
	2	1	44.1	5	207	6
X	3	2	50.0	1	296	3
	4	4	60.2	4	208	8
	5	5	62.5	3	263	8

The results shown in Table 2 indicate that Lot #3 ranked second in suppression of apoptotic cells, and first in induction of trypsin resistance. This sum of 3 was then determined to be the best of the five lots evaluated and it was then selected for use in the peptide assay described in Example 3.

Example 3

Assay of Putative Therapeutic Peptide Mixtures

In order to determine whether the assay described in Example 1 was effective in recognizing agents with potential therapeutic activity, the following assay was performed utilizing peptide mixtures. The assay was performed essentially as described in Example 1 with C3H-10T½ clone 8 with the addition of various peptide mixtures. The peptides utilized were obtained from soy beans and designated pp/g, EDI, ARD, FXP, GS90FB, pp750, SPH, PRO (commercially available from such sources as Arthur Daniels Midland and Purina); and a protein product obtained from peas and designated PEA commercially available from such companies as Grinsted and Novo Nordisk.

The cells were used during exponential growth phase when cell cycle position is randomly distributed with no cells arrested in G_0. At T=0, cultures were transferred to serum-free medium as an apoptosis stimulus. Controls included 1 nM TPA to ensure responsiveness of cell cultures, and acetone as a non-specific control solvent.

Peptide mixtures were added to serum-free medium at the highest concentration previously determined to exhibit no toxicity as determined by suppression of cloning efficiency in a conventional cytotoxicity assay. Toxicity was determined by detection of a statistically significant suppression of cell cloning efficiency when

the agents were added to cell cultures 24 h after seeding at 200 cells per 20 square centimeter culture plate. Assays were performed on 4 replicate culture plates and consisted of counting the number of clones of greater than 16 cells at day 7.

Only three peptide mixtures were found to be toxic at 1:10 dilution levels of the saturated stock solutions: pp750; SPH; and PRO. At 1:100 and 1:1000 dilution levels, none of the peptides were found to be toxic. Analyses of cell responses were made at either 24 hours (Figure 2 and Table 3) or 48 hours (Figure 3 and Table 4) after serum deprivation.

Three measurements were performed on each culture plate consisting of differential cell counts:

1. Apoptotic cell count: These released cells consisted of >95% apoptotic cells as shown by both ultrastructure analysis and DNA fragmentation analysis.

2. Proteinase resistant cell count: The fraction of cells that are resistant to proteinase-release is counted. These cells represent the subpopulation that responds to the apoptosis inhibitor TPA and contains the mitogenic responsive cells. Research indicates that this population is critical to control of apoptosis, probably through mechanisms related to modulation of gene expression through mechanisms related to cell shape and adhesion to substrates. This response has been shown to be related to but independent of simple suppression from apoptotic cell number. It remains a critical but empirical marker for apoptosis modulation.

3. Proteinase-sensitive cell count: This is the remaining fraction of the total cell population which exhibits high sensitivity to proteinase release from adhesion surface. At present this count is used to

calculate the total cell number in each plate in order to determine whether the agent had mitogenic properties.

The results are shown in Figures 2 and 3 and Tables 3-6.

Table 3

Experiment 158

Effects Determined at 24 hours

(SD)	medium control	solvent control	TPA 1 nM	sph	pro	g590-fb	pea
Apo	126.8(15.7)	114.2(23.1)	85.9(11.0)	130.9(15.7)	90.9(18.2)	122.5(27.0)	78.0(11.2)
PS	254.7(11.6)	253.3(2.6)	271.6(17.4)	222.2(31.7)	218.2(29.2)	196.9(25.9)	63.3(6.0)
PR	39.2(4.8)	36.2(4.0)	99.0(33.5)	66.0(5.9)	83.7(4.5)	83.7(16.2)	261.5(12.0)
Total	420.7	403.7	456.5	419.1	392.8	403.1	402.8

Table 4

Experiment 158

Effects determined at 48 hours

	medium	solvent	TPA 1 nM	sph	pro	g590-fb	pea	sph(+)	pro(+)	pea(+)	g590-fb(+)
Apo	126.4	105.1	54.1	100.2	79.2	34.8	141.3	89.8	62.1	103.2	58.2
PS	150.2	115.2	43.3	136.6	118.4	112.7	29.8	58.4	59.2	24.8	45.8
PR	92.5	105.8	306.7	72.6	96.1	103.6	178.4	242.2	232.8	240.7	248.4
Total	369.1	326.1	404.1	309.4	293.7	251.1	349.5	390.4	354.1	368.7	352.4

Table 5

Experiment 163

	control	acetone	SE90F	Alanate-166 1:1000	Alanate-166 1:1000	Alanate-166 1:100	TPA
Apo	14.1(1.1)	15.3(1.5)	10.8(2.3)	4.6(0.4)	9.2(0.7)	6.3(0.4)	21.7(0.7)
PS	27.1(1.5)	17.8(1.3)	27.8(8.5)	25.4(4.7)	22.3(5.2)	29.4(5.1)	19.6(2.3)
PR	39.8(3.7)	46.1(4.5)	31.3(0.6)	46.8(5.9)	43.8(1.7)	50.2(1.7)	61.1(5.2)
Total cells/ plate	81.3	79.2	69.9	76.8	75.3	85.9	102.4

Table 6
Experiment 157: Peptide mixtures in presence and absence of TPA
Cells per culture plate x 1000

+/- 1 nM TPA	Apo	PS	PR	Total	mitogenic	apo inhibition	PR activity
medium control	140.1	30.50	177.1	347.7			
solvent control	173.0	35.16	187.0	395.2			
TPA 1 nM	125.1	24.80	293.4	443.6	yes	yes	yes
pro(-)	91.3	35.60	162.5	289.4	no	yes	no
pro(+)	63.1	32.60	226.4	322.2			
pp750(-)	112.2	26.60	189.9	328.7	no	yes	no
pp750(+)	73.3	21.40	210.9	304.6			
g590-fb(-)	40.2	36.40	182.6	259.1	no	yes	no
g590-fb(+)	73.4	27.60	256.4	357.4			
pea(-)	92.0	20.40	182.8	295.2	no	yes	no
pea(+)	126.2	24.00	216.7	367.0			
pp16-10(-)	228.0	13.40	206.0	447.3	yes	no	no
pp16-10(+)	187.7	12.10	296.3	497.1			
fxp 720(-)	231.4	11.10	196.4	438.9	yes	no	no
fxp 720(+)	204.3	13.30	279.3	496.9			
ard(-)	201.9	16.30	206.7	424.9	yes	no	no
ard(+)	180.2	18.50	276.7	478.6			
edi(-)	151.5	19.20	146.5	317.2	no	no	no
edi(+)	131.0	17.60	205.1	353.6			

As determined by the results depicted in Tables 3-6 and Figures 2-4, the activities of the peptide mixtures were as follows;

1. No Activity. Defined as having no appreciable effect on any measurement at concentrations of 1:10, 1:100, and 1:1000. Peptide mixtures exhibiting no activity included SE90F and EDI;

2. Anti-apoptotic. Defined as being active at either 24 or 48 hours post serum deprivation in significantly depressing the number of apoptotic cells in each plate. Peptide mixtures exhibiting anti-apoptotic activity included: PRO (1:10, 1:100); pp750 (1:100); gs90fb (1:10); PEA (1:10); Alanate 166 (1:100, 1:1000); and Alanate 180 (1:100);

3. PR induction. Defined as able to induce significantly increased proteinase-resistant adhesion. Peptide mixtures exhibiting PR induction include: PEA (1:10); Alanate 180 (1:100); and SPH (1:1000); and

4. Mitogenic. Defined as able to significantly increase the total cell number 24 hours after addition to serum-free medium. Peptides exhibiting mitogenic activity include: pp16-10 (1:10); fxp-720 (1:10); and ARD (1:10).

It should be noted that only PEA and alanate-180 were found to have activity in two measurements, Anti-apoptotic activity and PR induction activity. Although neither was mitogenic, PEA also did not interfere with TPA mitogenicity when added in combination.

It has also been found that agents may effect apoptosis at 24 or 48 hours. Consequently, it is preferable to analyze the effect of putative therapeutic agents at both 24 and 48 hours.

Example 4

Statistical Analysis of Screening Assay

In order to determine the requisite sample size to assure statistically significant results, the results presented in Tables 3 and 4 were recompiled and subject to statistical analysis. The statistical analysis was performed as described in Daniel, Biostatistics: A Foundation in the Health Sciences, 2 ed. John Wiley and Sons (1978). The results of the assessment of statistical significance of sample size are presented in Table 7.

Typical results were used including mean values and standard deviation of the means from actual assay measurements obtained as previously described by Daniel (1978). Mean values of both control and peptide-treated groups and their respective standard deviations are tabulated followed by sample sizes of either 2 or 4 replicate culture plates. Based upon choices of culture replicate values of either 2 or 4, statistics were calculated and levels of significance were determined. The levels of significance were evaluated by selecting the lowest value of replicates that provided statistical reliability as determined by p values of 0.050 or less in order to provide general statistical reliability at the 95% confidence level.

In Table 7, examples 1-12 are results obtained with G590-FB at 24 hours (1-6) and 48 hours (7-12) respectively; examples 13-24 are those obtained with TPA at 1 nM at 24 hours (13-18) and 48 hours (19-24) respectively; and examples 25-36 are those obtained with PEA proteins at 24 hours (25-30) and 48 hours (31-36) respectively.

Table 7

STATISTICAL ASSESSMENT OF METHODOLOGIES TO IDENTIFY POSSIBLE APOPTOSIS MODULATING PEPTIDES

	SAMPLE CONTROL MEAN	PEPTIDE MEAN	CONTROL SD	PEPTIDE SD	CONTROL SAMPLE SIZE	PEPTIDE SAMPLE SIZE	EQUAL VARIANCE TEST: F STAT	EQUAL VARIANCE TEST: P-VALUE	POOLED VARIANCE	TEST	T STAT	DF	P-VALUE
1.	126.8	122.5	15.7	27	2	2	2.95752	0.67060	487.745	=	0.19470	2.00000	0.86361
										≠	0.19470	1.60686	0.86738
2.	126.8	122.5	15.7	27	4	4	2.95752	0.39693	487.745	=	0.27535	6.00000	0.79228
										≠	0.27535	4.82058	0.79446
3.	254.7	196.9	11.6	25.9	4	4	4.98521	0.21988	402.685	=	4.07343	6.00000	0.006550
										≠	4.07343	4.15700	0.014041
4.	254.7	196.9	11.6	25.9	2	2	4.98521	0.53614	402.685	=	2.88035	2.00000	0.10236
										≠	2.88035	1.38567	0.15346
5.	39.2	83.7	4.8	16.2	4	4	11.3906	0.075934	142.74	=	-5.26747	6.00000	.0018878
										≠	-5.26747	3.52272	.0087946

	SAMPLE CONTROL MEAN	PEPTIDE MEAN	CONTROL SD	PEPTIDE SD	CONTROL SAMPLE SIZE	PEPTIDE SAMPLE SIZE	EQUAL VARIANCE TEST: F STAT	EQUAL VARIANCE TEST: P-VALUE	POOLED VARIANCE	TEST	T STAT	DF	P-VALUE
6.	39.2	83.7	4.8	16.2	2	2	11.3906	0.36676	142.74	=	-3.72466	2.00000	0.06512
										≠	-3.72466	1.17424	0.13736
7.	126.4	34.8	15.6505	7.67020	4	4	4.16333	0.27195	151.885	=	10.5112	6.00000	.00004356
										≠	10.5112	4.36254	.00029166
8.	126.4	34.8	15.6505	7.67020	2	2	4.16333	0.58020	151.885	=	7.43256	2.00000	0.017625
										≠	7.43256	1.45418	0.039426
9.	150.2	112.7	6.84068	14.8244	4	4	4.69632	0.23614	133.279	=	4.59372	6.00000	.0037165
										≠	4.59372	4.22218	.0088637
10.	150.2	112.7	6.84068	14.8244	2	2	4.69632	0.55046	133.279	=	3.24825	2.00000	0.08313
										≠	3.24825	1.40739	0.12950
11.	92.5	103.6	11.3265	20.0516	4	4	3.13404	0.37322	285.179	=	-0.96398	6.00000	0.37228
										≠	-0.96398	4.73756	0.38164

	SAMPLE CONTROL MEAN	PEPTIDE MEAN	CONTROL SD	PEPTIDE SD	CONTROL SAMPLE SIZE	PEPTIDE SAMPLE SIZE	EQUAL VARIANCE TEST: F STAT	EQUAL VARIANCE TEST: P-VALUE	POOLED VARIANCE	TEST	T STAT	DF	P-VALUE
12.	92.5	103.6	11.3265	20.0516	2	2	3.13404	0.65468	265.179	=	-0.68164	2.00000	0.5658 1
										≠	-0.68164	1.57919	0.5815 4
13.	254.7	271.6	11.6	17.4	2	2	2.25	0.74867	218.66	=	-1.14288	2.00000	0.3714 5
										≠	-1.14288	1.74227	0.3859 9
14.	254.7	271.6	11.6	17.4	4	4	2.25	0.52265	218.66	=	-1.61628	6.00000	0.1571 6
										≠	-1.61628	5.22680	0.1644 2
15.	39.2	99	4.8	33.5	2	2	48.7088	0.18120	572.645	=	-2.49896	2.00000	0.1297 0
										≠	-2.49896	1.04104	0.2343 4
16.	39.2	99	4.8	33.5	4	4	48.7088	.0096292	572.645	=	-3.53406	6.00000	
										≠	-3.53406	3.12313	0.0123 07 0.0361 29
17.	126.8	85.9	15.7	11	2	2	2.03711	0.77814	183.745	=	3.01728	2.00000	0.0945 3
										≠	3.01728	1.79114	0.1078 9

SAMPLE	CONTROL MEAN	PEPTIDE MEAN	CONTROL SD	PEPTIDE SD	CONTROL SAMPLE SIZE	PEPTIDE SAMPLE SIZE	EQUAL VARIANCE TEST: F STAT	EQUAL VARIANCE TEST: P-VALUE	POOLED VARIANCE	TEST	T STAT	DF	P-VALUE
18.	126.8	85.9	15.7	11	4	4	2.03711	0.57382	183.745	=	4.26708	6.00000	.00528 00
										≠	4.26708	5.37342	.00677 53
19.	126.4	54.1	15.6505	6.92782	4	4	5.10342	0.21376	146.466	=	8.44860	6.00000	.00015 014
										≠	8.44860	4.13221	.00093 096
20.	126.4	54.1	15.6505	6.92782	2	2	5.10342	0.53060	146.466	=	5.97406	2.00000	0.0268 94
										≠	5.97406	1.37740	0.0598 35
21.	150.2	43.3	6.84068	2.77401	4	4	6.08111	0.17235	27.2450	=	28.9634	6.00000	.00000 01122
										≠	28.9634	3.96068	.00000 92557
22.	150.2	43.3	6.84068	2.77401	2	2	6.08111	0.49052	27.2450	=	20.4802	2.00000	0.0023 76
										≠	20.4802	1.32023	0.0129 57
23.	92.5	306.7	11.3265	103.782	4	4	83.9562	.0043206	5449.53	=	-4.10350	6.00000	0.0063 32
										≠	-4.10350	3.07146	0.0250 32

	SAMPLE CONTROL MEAN	PEPTIDE MEAN	CONTROL SD	PEPTIDE SD	CONTROL SAMPLE SIZE	PEPTIDE SAMPLE SIZE	EQUAL VARIANCE TEST: F STAT	EQUAL VARIANCE TEST: P-VALUE	POOLED VARIANCE	TEST	T STAT	DF	P-VALUE
24.	92.5	306.7	11.3265	103.782	2	2	83.9562	0.13841	5449.53	=	-2.90162	2.00000	0.10108
										≠	-2.90162	1.02382	0.20658
25.	126.8	78	15.7	11.2	4	4	1.96500	0.59304	185.965	=	5.06080	6.00000	.0023089
										≠	5.06080	5.42531	.0030906
26.	126.8	78	15.7	11.2	2	2	1.96500	0.78896	185.965	=	3.57853	2.00000	0.069990
										≠	3.57853	1.80844	0.080999
27.	254.7	63.3	11.6	6	4	4	3.73778	0.30754	85.28	=	29.3112	6.00000	.0000001045
										≠	29.3112	4.49801	.0000026126
28.	254.7	63.3	11.6	6	2	2	3.73778	0.60778	85.28	=	20.7261	2.00000	.0023198
										≠	20.7261	1.49934	.0079914
29.	39.2	261.5	4.8	12	4	4	6.25	0.16654	83.52	=	-34.4001	6.00000	.0000000402
										≠	-34.4001	3.93604	.0000049874

	SAMPLE CONTROL MEAN	PEPTIDE MEAN	CONTROL SD	PEPTIDE SD	CONTROL SAMPLE SIZE	PEPTIDE SAMPLE SIZE	EQUAL VARIANCE TEST: F STAT	EQUAL VARIANCE TEST: P-VALUE	POOLED VARIANCE	TEST	T STAT	DF	P-VALUE
30.	39.2	261.5	4.8	12	2	2	6.25	0.48448	83.52	=	-24.3245	2.00000	0.0016 86
										≠	-24.3245	1.31201	0.0105 70
31.	126.4	141.3	15.6505	20.2892	4	4	1.68065	0.68022	328.295	=	-1.16297	6.00000	0.2890 0
										≠	-1.16297	5.63660	0.2917 0
32.	126.4	141.3	15.6505	20.2892	2	2	1.68065	0.83657	328.295	=	-0.82235	2.00000	0.4973 2
										≠	-0.82235	1.87887	0.5020 9
33.	150.2	29.8	6.84068	2.82464	4	4	5.86503	0.18029	27.3867	=	32.5365	6.00000	.00000 00561
										≠	32.5365	3.99411	.00000 53961
34.	150.2	29.8	6.84068	2.82464	2	2	5.86503	0.49859	27.3867	=	23.0068	2.00000	0.0018 84
										≠	23.0068	1.33137	0.0107 79
35.	92.5	178.4	11.3265	8.18662	4	4	1.91419	0.60723	97.6555	=	-12.2931	6.00000	.00001 7658
										≠	-12.2931	5.46244	.00003 4574

	SAMPLE CONTROL MEAN	PEPTIDE MEAN	CONTROL SD	PEPTIDE SD	CONTROL SAMPLE SIZE	PEPTIDE SAMPLE SIZE	EQUAL VARIANCE TEST: F STAT	EQUAL VARIANCE TEST: P-VALUE	POOLED VARIANCE	TEST	T STAT	DF	P-VALUE
36.	92.5	178.4	11.3265	8.18662	2	2	1.91419	0.79686	97.6555	=	-8.69250	2.00000	0.012978
										≠	-8.69250	1.82081	0.017160

The results obtained from the statistical analysis indicate that the sample size is critical to obtaining statistically significant results. Changing the sample number from 4 to 2 results in a statistically significant change in both the P and T values. Thus, it is preferred that a sample size of at least 4 be utilized in order to obtain statistically significant results.

Example 5

Cell Cultivation and Induction of Cell Death in Exponential and Quiescent Phase Cells

In order to compare the differential effect of apoptosis inducing agents on exponential and quiescent phase cells, the following experiments were performed. C3H-10T½ (clone 8) cells were grown in basal medium Eagle's (BME) medium supplemented with 10% heat-inactivated FBS at 37°C in a humidified 5% CO_2 atmosphere without antibiotics. Cells were seeded at 2 x 10^3 cells/ml and fed every 3-4 days. After 1 week the cells were in late log phase, completely confluent but still demonstrating occasional mitotic cells. Approximately 2 weeks after the initial seeding, the cells were completely confluent and few if any mitotic cells are present. These are the quiescent cells used in this and the following Example.

Apoptotic death of exponentially growing C3H-10T½ cells is usually induced by serum deprivation. As described in Example 1, exponentially proliferating cultures at approximately 75% confluency are transferred to serum-free medium. At 24 hours, the apoptotic (i.e., non-adherent) cells and the non-apoptotic (i.e., adherent) cells were collected separately for further analysis. Similar steps were performed with quiescent cells. To analyze the effect of cycloheximide other agents on apoptosis in cells at different stages of the

cell cycle, these agents were added to exponentially growing and quiescent cells both in the presence and in the absence of serum. The results obtained are presented in Figures 5-8.

Measurement of Cell Viability

Adherent and nonadherent cells were measured as described in Example 1.

As described in Example 1, exponentially growing C3H-10T½ cells are very sensitive to serum deprivation. Fig. 5a shows that by 24 hours in serum-free medium about 50% of the cells die by apoptosis. Cycloheximide (10 μg/ml) delays the death of exponentially growing C3H-10T½ cells induced by serum deprivation (Fig. 6) and TPA seriously inhibits cell death. Quiescent C3H-10T½ cells are much more resistant to depletion of serum but die very rapidly in the presence of cycloheximide (Fig. 5b); however, TPA does not prevent death of quiescent cells induced by cycloheximide. Fig. 7 shows how in going from the exponential phase to quiescent phase the sensitivity to serum deprivation decreases and sensitivity to cycloheximide increases. (During cultivation each day cells were taken to measure their sensitivity to these two factors).

To be sure that death of the quiescent cells was induced by cycloheximide is due to inhibition of protein synthesis and not by side effects of the drug, the influence of different inhibitors of RNA and protein synthesis was analyzed. Fig. 8 shows that not only cycloheximide but also puromycin, actinomycin, and α-amanatin in the concentrations shown in Figure 8, induce death of quiescent C3H-10T½ cells. Thus, the inhibition of RNA or protein synthesis induces apoptosis in quiescent C3H-10T½ cells.

Thus, the combination of exponentially growing and quiescent cells is much more effective for analysis of both apoptosis inducing and inhibiting factors.

Example 6

Apoptosis of Rat Cardiomyocytes

The following experiment was performed in order to determine the correlation between the differential effect of apoptosis inducing agents on C3H-10T½ and terminally differentiated rat cardiomyocytes.

Neonatal rat cardiomyocytes were isolated and cultivated according to Simpson, Circ. Res. 56:884-894 (1985).

Rat neonatal cardiomyocytes (6.0 x 10^5/ml) in MEM containing 5% FBS were seeded in 6- or 12-well plates, or in 6 cm dishes. The next day all nonadherent cells (normally about 25-35% of the initial amount of cardiomyocytes) were washed out and the remaining cells were cultivated in the same medium. The final density of cardiomyocytes was about (7-8) x 10^4/cm^2. Cycloheximide at a final concentration of 10 μg/ml, treatment was performed in MEM in the presence or in the absence of serum. In the absence of cycloheximide, cardiomyocyte death was insignificant during 35 hours. The kinetics of cardiomyocyte death were analyzed at 10 μg cycloheximide per ml at 24 and 36 hours in the presence and absence of serum. The data are presented in Figure 9. A dose response curve is also presented in Figure 10.

The data obtained with neonatal rat cardiomyocytes indicate that in many respects these cells are more similar to quiescent rather than exponentially growing C3H-10T½ cells. The results obtained showed that upon serum deprivation for 36 hours 0-2% of cardiomyocytes died but the addition of cycloheximide (10 μg/ml final concentration) increased the amount of dead

cells up to 15 and 36% in the presence and absence of serum, respectively.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be considered as limiting the scope of the invention, which is delineated by the appended claims.

Claims

1. A method of identifying potentially therapeutically effective agents comprising assessing the ability of the agents to alter the proteinase sensitivity of apoptosis sensitive cells exposed to the agents.

2. A method of screening agents for potential therapeutic efficacy comprising the steps of:

a. providing a cell strain that is sensitive to apoptotic agents;

b. culturing the cell strain for a time and under conditions sufficient to attain exponential proliferation without density dependent constraints;

c. exposing the cell strain to conditions that are known to induce apoptosis;

d. exposing the cell strain to an agent to be screened;

e. culturing the cell strain for a time and under conditions suitable to maintain exponential proliferation without density dependent constraints;

f. removing and enumerating cells that display diminished adherence to yield adherent cells;

g. exposing the adherent cells to a proteinase for a time and under conditions suitable to remove proteinase sensitive cells from the surface;

h. incubating the adherent cells for a time and under conditions suitable to release proteinase sensitive cells;

i. removing and enumerating the proteinase sensitive cells to yield proteinase resistant cells;

j. incubating the proteinase resistant cells for a time and under conditions suitable to release the cells; and

k. collecting and enumerating the proteinase resistant cells;

wherein the agent is determined to have potential therapeutic efficacy if the number of non-adherent cells is reduced relative to the number of non-adherent cells in the control, and the number of proteinase resistant cells increases relative to a control.

3. The method according to claim 2 wherein the cell strain is a pluripotent embryonic cell with a stable, normal, intact, phenotype.

4. The method according to claim 2 wherein the cell strain exhibits density dependent regulation of proliferation and death; is pluripotent and capable of demonstrating at least two distinct differentiated cell types upon suitable stimulus; is sensitive to loss due to transformation induced by carcinogenic and oncogenic agents; responds to agents that under suitable conditions both induce and block proliferation, differentiation, and apoptosis; and exhibits apoptotic cell death.

5. The method according to claim 4 wherein apoptosis of the cell strain is marked by ultrastructural changes of chromatin and specific changes in the molecular structure of DNA associated with apoptotic DNA degradation.

6. The method according to claim 2 wherein the cell strain is C3H-10T½ clone 8.

7. The method according to claim 2 wherein the initial culturing step to attain stable exponential proliferation without density dependent constraints is for about 5 days

at 37°C with a gas overlay of 5% CO_2 in a suitable growth medium.

8. The method according to claim 2 wherein the condition that induces apoptosis is serum deprivation, growth factor deprivation, ultraviolet radiation, γ-radiation, soft β-radiation, hypo-osmotic shock, chemotherapeutic agents or specific receptor mediated agents.

9. The method according to claim 8 wherein the culturing in step (e) continues for a time and under conditions suitable to attain a density of about one population density doubling from density-dependent arrest of cell cycle.

10. The method according to claim 9 wherein the culturing is for about 5 days at 37°C with 5% CO_2.

11. The method according to claim 2 wherein the proteinase is trypsin.

12. The method according to claim 2 wherein the adherent cells are incubated in the presence of concentrations of about 1 μg/ml to 1 mg/ml trypsin for about 10 minutes with gentle shaking at room temperature and are removed by gentle shearing effected by washing.

13. The method according to claim 2 wherein the proteinase resistant cells are released by incubation in the presence of an agent that decreases the calcium concentration to a level sufficient to release the cells.

14. The method according to claim 13 wherein the agent that decreases the calcium concentration is a calcium chelator.

15. The method according to claim 14 wherein the calcium chelator is selected from the group consisting of ethylenediaminetetraacetic acid and ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetraacetic acid.

16. A method of screening agents for potential therapeutic efficacy comprising the steps of:

a. culturing the cell strain C3H-10T½ for a time and under conditions sufficient to attain stable exponential proliferation without density dependent constraints at any time during the assay;

b. exposing the cell strain to conditions that are known to induce apoptosis;

c. exposing the cell strain to an agent to be screened simultaneously with apoptosis induction or shortly thereafter;

d. culturing the cell strain for a time and under conditions suitable to maintain a density of about one population doubling from density-dependent arrest of cell cycle;

e. removing and enumerating cells that display diminished adherence to yield adherent cells;

f. exposing the adherent cells to a proteinase;

g. incubating the adherent cells for a time and under conditions suitable to release proteinase sensitive cells;

h. removing and enumerating the proteinase sensitive cells to yield proteinase resistant cells;

i. incubating the proteinase resistant cells for a time and under conditions suitable to release the cells; and

j. collecting and enumerating the proteinase resistant cells;

wherein the agent is determined to have potential therapeutic efficacy if the ratio of proteinase sensitive cells to proteinase resistant cells changes relative to a control.

17. A method of obtaining a cell strain that retains responsiveness to apoptosis modulating agents comprising the steps of:

a. obtaining a cell sample;

b. culturing the cell sample for a time and under conditions sufficient to attain exponential proliferation without density dependent constraints at any time during the method;

c. exposing the cell sample to conditions that induce apoptosis; simultaneously treating the cell sample with an agent known to induce proteinase resistance;

d. culturing the cell sample for a time and under conditions suitable to maintain exponential proliferation without density dependent constraints;

e. removing cells that display diminished adherence to yield adherent cells;

f. exposing the adherent cells to a proteinase;

g. incubating the adherent cells for a time and under conditions suitable to release proteinase sensitive cells;

h. collecting the remaining proteinase resistant cells;

i. collecting the proteinase resistant cells that are then retained and passaged to generate a stable cell strain that continues to express responsiveness to apoptosis inducers and inhibitors.

18. The method according to claim 17 wherein the cell sample is a pluripotent embryonic cell with a normal, intact, phenotype.

19. The method according to claim 17 wherein the cell sample of step (a) is cultured for about 5 days at 37°C with an atmosphere of 5% CO_2 to attain stable exponential proliferation without density dependent constraints.

20. The method according to claim 17 wherein the condition that induces apoptosis is serum deprivation, growth factor deprivation, ultraviolet radiation, γ-radiation, soft β-radiation, hypo-osmotic shock, chemotherapeutic agents or specific receptor mediated agents.

21. The method according to claim 17 wherein the culturing in step (d) continues for a time and under conditions suitable to attain a density of about one population density doubling from density-dependent arrest of cell cycle.

22. The method according to claim 21 wherein the culturing is for about 5 days at 37°C with 5% CO_2 to attain exponential proliferation without density dependent constraints.

23. The method according to claim 17 wherein the proteinase is trypsin.

24. The method according to claim 17 wherein the adherent cells are incubated in the presence of about 1 μg/ml to 1 mg/ml trypsin for about 10 minutes with gentle shaking at room temperature and are removed by a gentle shearing effected by washing.

25. The method according to claim 17 wherein the proteinase resistant cells are released by incubation in the presence of an agent that decreases the calcium concentration to a level sufficient to release the cells.

26. The method according to claim 25 wherein the agent that decreases the calcium concentration is a calcium chelator.

27. The method according to claim 25 wherein the calcium chelator is selected from the group consisting of ethylenediaminetetraacetic acid and ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetraacetic acid.

28. A cell strain obtained by the method according to claim 17.

29. A method of preserving cellular responsiveness to apoptosis modulating agents comprising the steps of:

a. obtaining a cell strain that is initially responsive to apoptotic agents;

b. culturing the cell strain for a time and under conditions sufficient to attain stable exponential proliferation without density dependent constraints at any time during the method;

c. exposing the cell strain to conditions that induce apoptosis;

d. culturing the cell strain for a time and under conditions suitable to maintain exponential proliferation without density dependent constraints;

e. removing cells that display diminished adherence to yield adherent cells;

f. exposing the adherent cells to a proteinase;

g. incubating the adherent cells for a time and under conditions suitable to release proteinase sensitive cells; and

h. collecting the released proteinase sensitive cells to obtain a cell strain that is responsive to apoptotic agents.

30. The method according to claim 29 wherein the cell strain is a pluripotent embryonic cell with a normal, stable, intact, phenotype.

31. The method according to claim 29 wherein the cell strain exhibits density dependent regulation of proliferation and death; is pluripotent and capable of demonstrating at least two distinct differentiated cell types upon suitable stimulus; is sensitive to loss due to transformation induced by carcinogenic and oncogenic agents; responds to agents that under suitable conditions both induce and block proliferation, differentiation, and apoptosis; and exhibits apoptotic cell death.

32. The method according to claim 30 wherein apoptosis of the cell strain is marked by ultrastructural changes of chromatin and specific changes in the molecular structure of DNA associated with apoptotic DNA degradation.

33. The method according to claim 31 wherein the cell strain is C3H-10T½ clone 8.

34. The method according to claim 29 wherein the initial culturing step to attain exponential proliferation without density dependent constraints is for about 5 days at 37°C with a gas overlay of 5% CO_2 in a suitable growth medium.

35. The method according to claim 29 wherein the condition that induces apoptosis is serum deprivation, growth factor deprivation, ultraviolet radiation, γ-radiation, soft β-radiation, hypo-osmotic shock, chemotherapeutic agents or specific receptor mediated agents.

36. The method according to claim 28 wherein the culturing in step (d) continues for a time and under conditions suitable to attain a density of about one population doubling from density-dependent arrest of cell cycle.

37. The method according to claim 29 wherein the culturing is for about 5 days at 37°C with 5% CO_2.

38. The method according to claim 28 wherein the proteinase is trypsin.

39. The method according to claim 29 wherein the adherent cells are incubated in the presence of about 1 μg/ml to 1 mg/ml trypsin for about 10 minutes with shaking at room temperature and are removed by gentle shearing effected by washing.

40. The method according to claim 29 wherein the proteinase resistant cells are released by incubation in the presence of an agent that decreases the calcium concentration to a level sufficient to release the cells.

41. The method according to claim 29 wherein the agent that decreases the calcium concentration is a calcium chelator.

42. The method according to claim 29 wherein the calcium chelator is selected from the group consisting of ethylenediaminetetraacetic acid and ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetraacetic acid.

43. A method of preserving cellular responsiveness to apoptosis modulating agents comprising the steps of:

a. culturing cell strain C3H-10T½ for a time and under conditions sufficient to attain stable exponential proliferation without density dependent restraints at any time during the assay;

b. exposing the cell strain to conditions that induce apoptosis;

c. culturing the cell strain for a time and under conditions suitable to attain a density of about one population doubling from entering G1 phase;

d. removing cells that display diminished adherence to yield adherent cells;

e. exposing the adherent cells to a proteinase;

f. incubating the adherent cells for a time and under conditions suitable to release proteinase sensitive cells; and

g. collecting the released proteinase sensitive cells to obtain a cell strain that is responsive to apoptotic agents.

44. A method of screening agents for potential therapeutic efficacy comprising the steps of:

a. providing a cell strain that is sensitive to apoptotic agents;

b. culturing the cell strain for a time and under conditions sufficient to attain quiescence;

c. optionally exposing the cell strain to conditions that are known to induce apoptosis;

d. exposing the cell strain to an agent to be screened;

e. culturing the cell strain for a time and under conditions suitable to maintain quiescence;

f. removing and enumerating cells that display diminished adherence to yield adherent cells;

g. exposing the adherent cells to a proteinase for a time and under conditions suitable to remove proteinase sensitive cells from the surface;

h. incubating the adherent cells for a time and under conditions suitable to release proteinase sensitive cells;

i. removing and enumerating the proteinase sensitive cells to yield proteinase resistant cells;

j. incubating the proteinase resistant cells for a time and under conditions suitable to release the cells; and

k. collecting and enumerating the proteinase resistant cells;

wherein the agent is determined to have potential therapeutic efficacy if the number of non-adherent cells is reduced relative to the number of non-adherent cells in the control, and the number of proteinase resistant cells increases relative to a control.

45. The method according to claim 44 wherein the cell strain is a pluripotent embryonic cell with a stable, normal, intact, phenotype.

46. The method according to claim 44 wherein the cell strain exhibits density dependent regulation of proliferation and death; is pluripotent and capable of

demonstrating at least two distinct differentiated cell types upon suitable stimulus; is sensitive to loss due to transformation induced by carcinogenic and oncogenic agents; responds to agents that under suitable conditions both induce and block proliferation, differentiation, and apoptosis; and exhibits apoptotic cell death.

47. The method according to claim 46 wherein apoptosis of the cell strain is marked by ultrastructural changes of chromatin and specific changes in the molecular structure of DNA associated with apoptotic DNA degradation.

48. The method according to claim 44 wherein the cell strain is C3H-10T½ clone 8.

49. The method according to claim 44 wherein the initial culturing step to attain quiescence is for about 5 days at 37°C with a gas overlay of 5% CO_2 in a suitable growth medium.

50. The method according to claim 44 wherein the condition that induces apoptosis is serum deprivation, growth factor deprivation, ultraviolet radiation, γ-radiation, soft β-radiation, hypo-osmotic shock, chemotherapeutic agents or specific receptor mediated agents.

51. The method according to claim 50 wherein the culturing is for about 5 days at 37°C with 5% CO_2.

52. The method according to claim 44 wherein the proteinase is trypsin.

53. The method according to claim 44 wherein the adherent cells are incubated in the presence of concentrations of about 1 μg/ml to 1 mg/ml trypsin for about 10 minutes with gentle shaking at room temperature and are removed by gentle shearing effected by washing.

54. The method according to claim 44 wherein the proteinase resistant cells are released by incubation in the presence of an agent that decreases the calcium concentration to a level sufficient to release the cells.

55. The method according to claim 54 wherein the agent that decreases the calcium concentration is a calcium chelator.

56. The method according to claim 55 wherein the calcium chelator is selected from the group consisting of ethylenediaminetetraacetic acid and ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetraacetic acid.

57. A method of screening agents for potential therapeutic efficacy comprising the steps of:

a. culturing the cell strain C3H-10T½ for a time and under conditions sufficient to attain quiescence;

b. optionally exposing the cell strain to conditions that are known to induce apoptosis;

c. exposing the cell strain to an agent to be screened simultaneously with apoptosis induction or shortly thereafter;

d. culturing the cell strain for a time and under conditions suitable to maintain quiescence;

e. removing and enumerating cells that display diminished adherence to yield adherent cells;

f. exposing the adherent cells to a proteinase;

g. incubating the adherent cells for a time and under conditions suitable to release proteinase sensitive cells;

h. removing and enumerating the proteinase sensitive cells to yield proteinase resistant cells;

i. incubating the proteinase resistant cells for a time and under conditions suitable to release the cells; and

j. collecting and enumerating the proteinase resistant cells;

wherein the agent is determined to have potential therapeutic efficacy if the ratio of proteinase sensitive cells to proteinase resistant cells changes relative to a control.

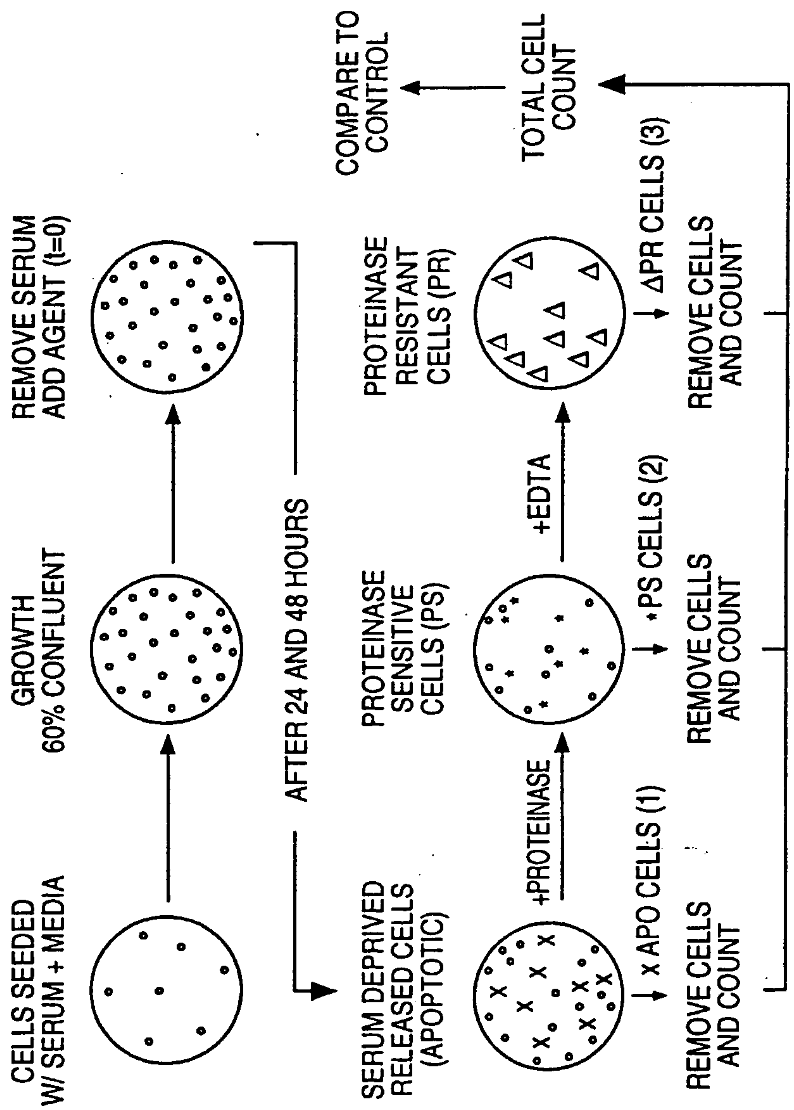

CELLS SEEDED
W/ SERUM + MEDIA
GROWTH
60% CONFLUENT
REMOVE SERUM
ADD AGENT (t=0)
AFTER 24 AND 48 HOURS
SERUM DEPRIVED
RELEASED CELLS
(APOPTOTIC)
+PROTEINASE
PROTEINASE
SENSITIVE
CELLS (PS)
+EDTA
PROTEINASE
RESISTANT
CELLS (PR)
COMPARE TO
CONTROL
TOTAL CELL
COUNT
x APO CELLS (1)
*PS CELLS (2)
ΔPR CELLS (3)
REMOVE CELLS
AND COUNT
REMOVE CELLS
AND COUNT
REMOVE CELLS
AND COUNT

FIG. 1

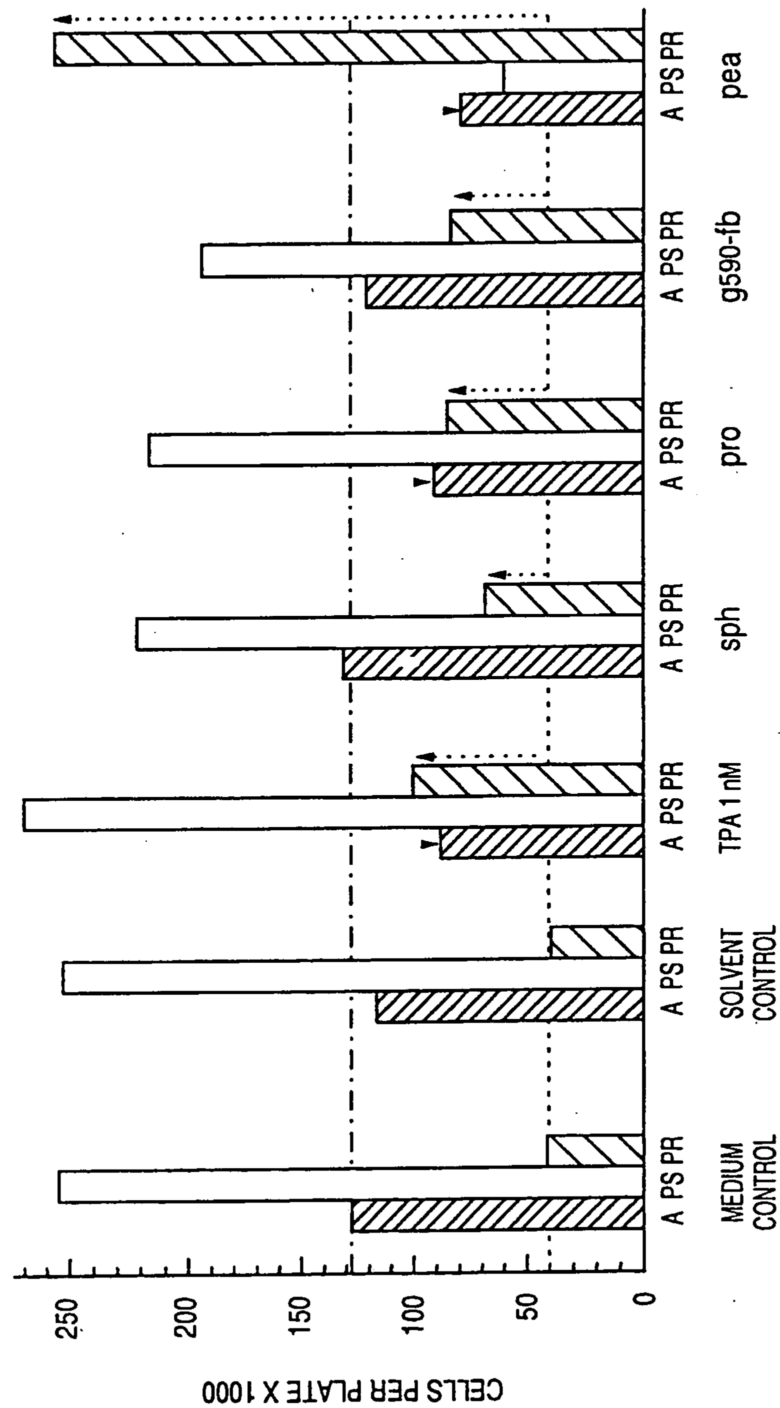

CELLS PER PLATE X 1000
0
50
100
150
200
250
A PS PR
MEDIUM CONTROL
SOLVENT CONTROL
TPA 1 nM
sph
pro
g590-fb
pea

FIG. 2

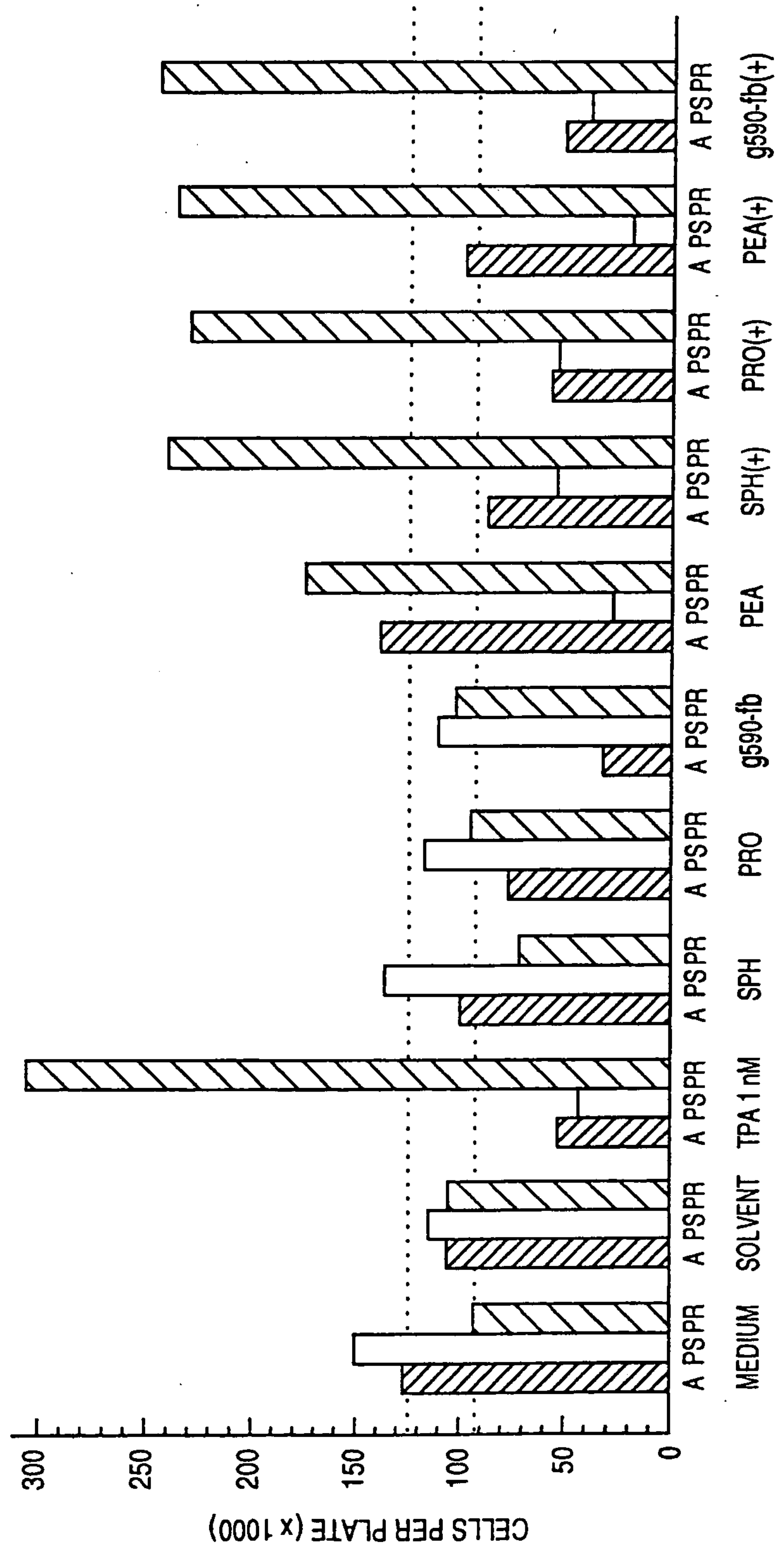

CELLS PER PLATE (x 1000)
0
50
100
150
200
250
300
A PSPR
MEDIUM
SOLVENT
TPA 1 nM
SPH
PRO
g590-fb
PEA
SPH(+)
PRO(+)
PEA(+)
g590-fb(+)

FIG. 3

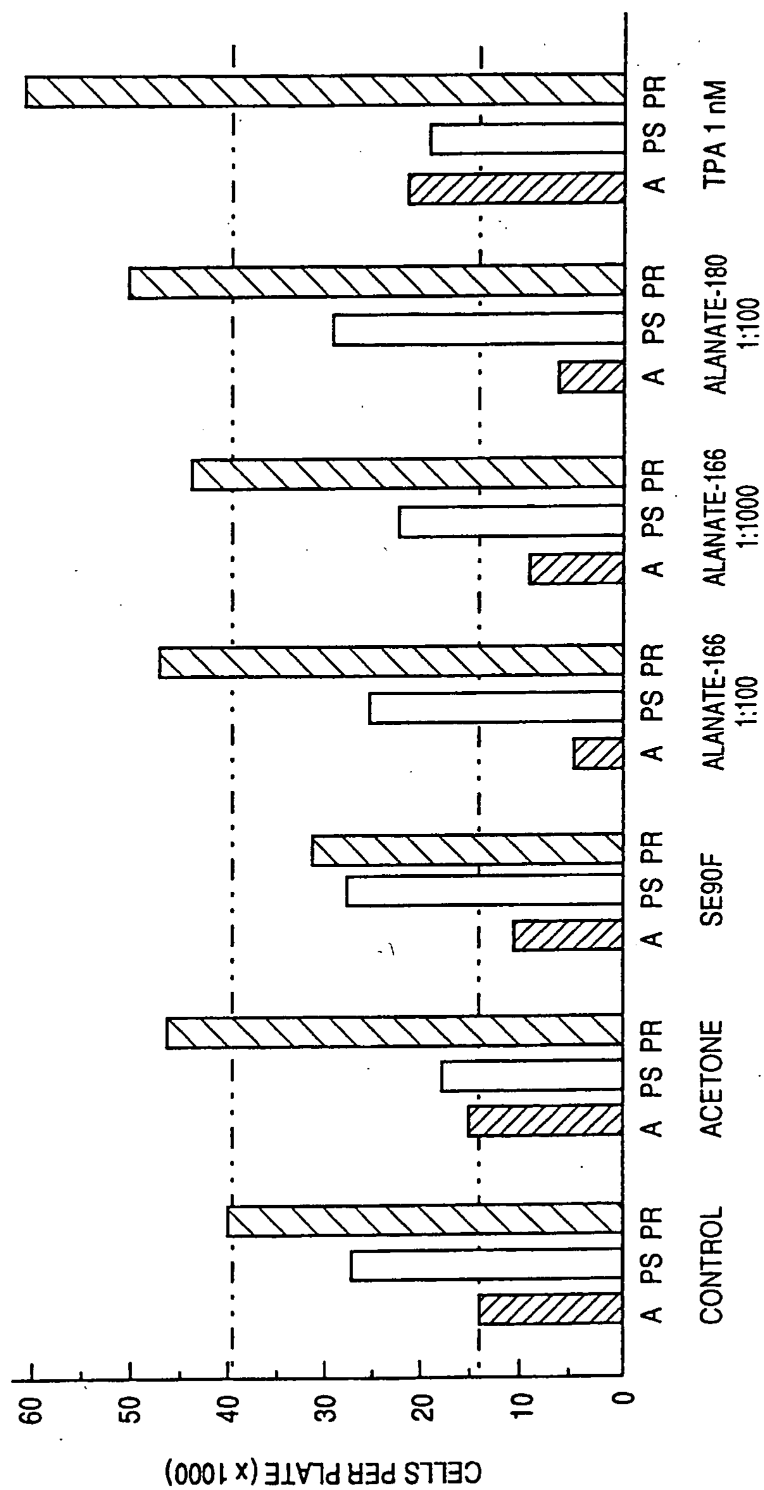

CELLS PER PLATE (x 1000)
0
10
20
30
40
50
60
A PS PR
CONTROL
A PS PR
ACETONE
A PS PR
SE90F
A PS PR
ALANATE-166
1:100
A PS PR
ALANATE-166
1:1000
A PS PR
ALANATE-180
1:100
A PS PR
TPA 1 nM

FIG. 4

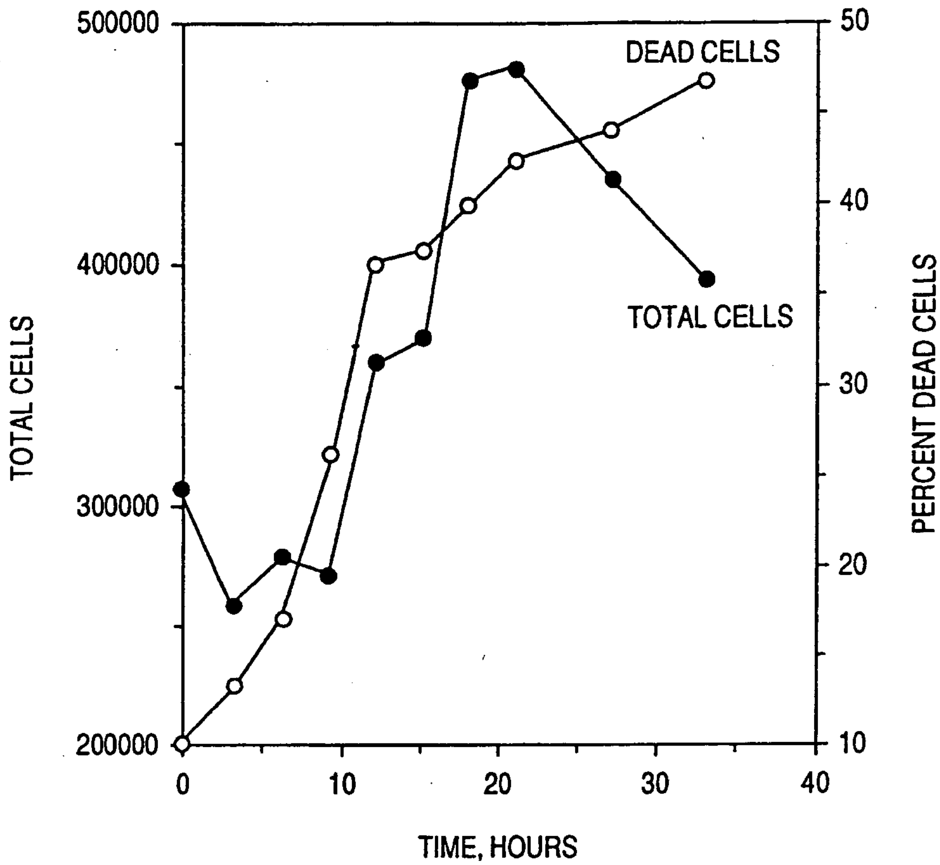

500000
400000
300000
200000
TOTAL CELLS
50
40
30
20
10
PERCENT DEAD CELLS
DEAD CELLS
TOTAL CELLS
0
10
20
30
40
TIME, HOURS

FIG. 5A

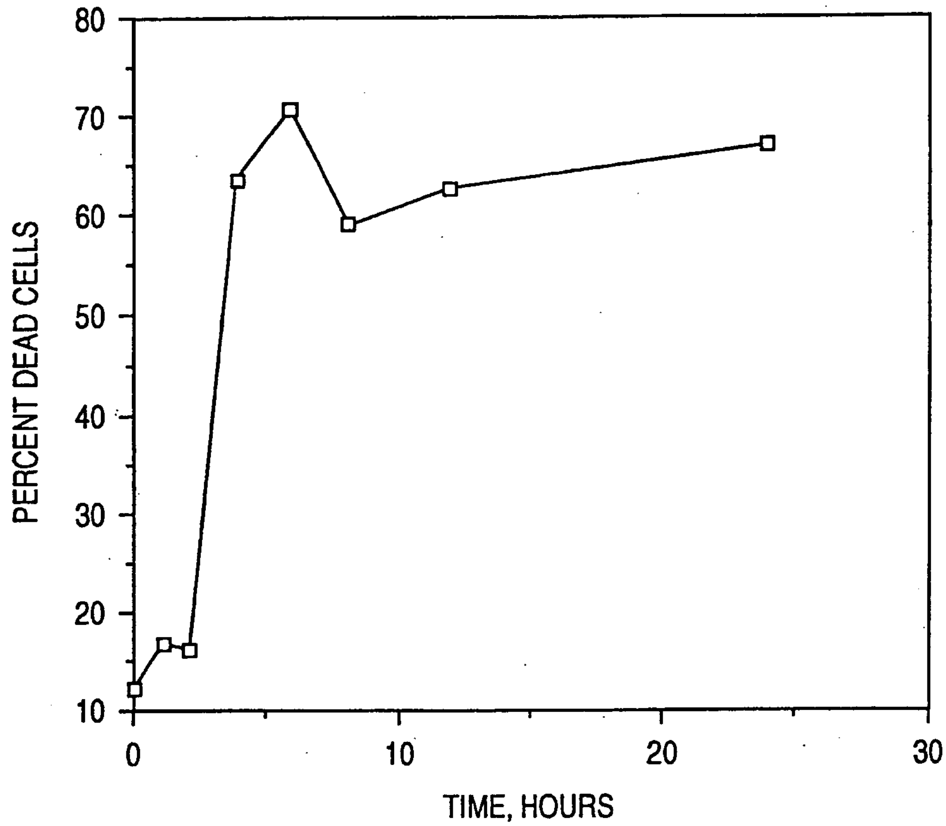


FIG. 5B

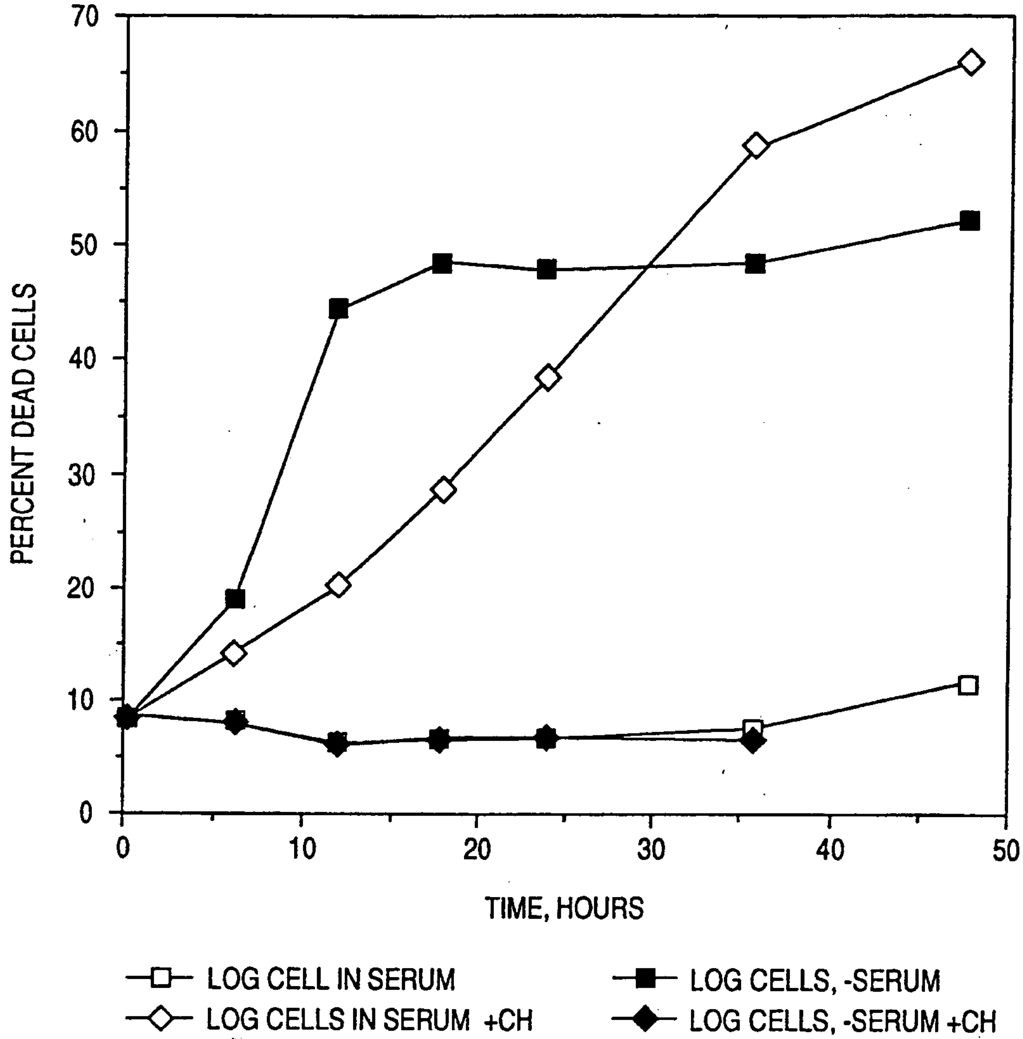
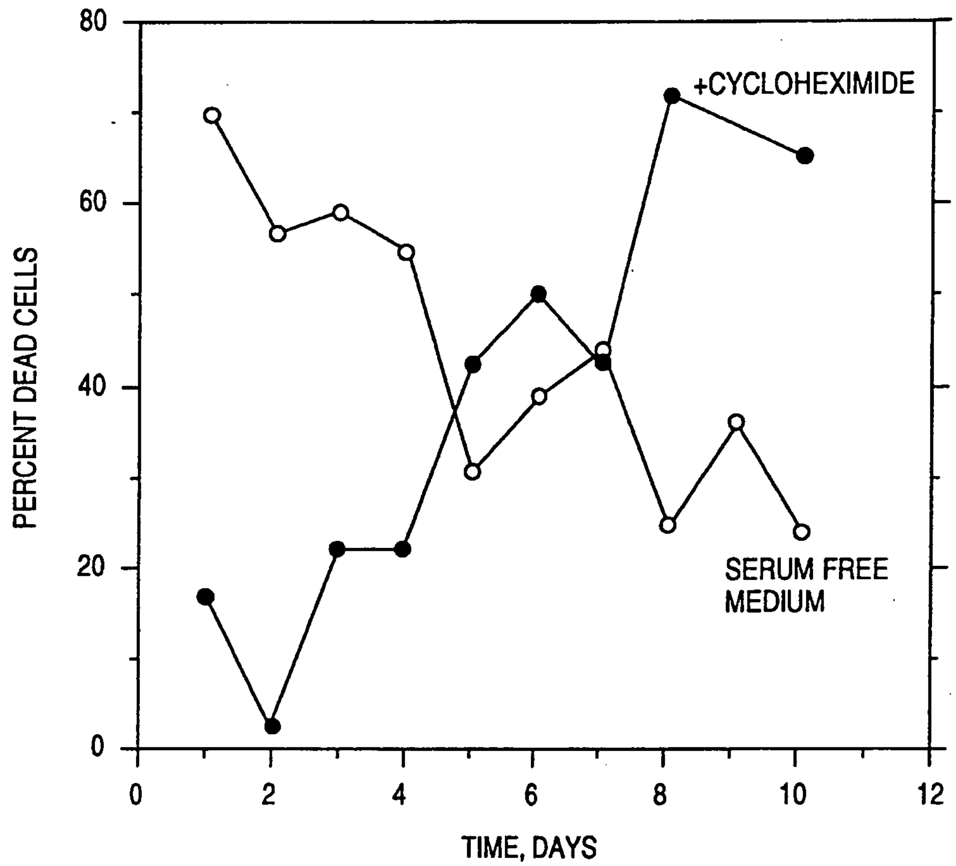

70
60
50
40
30
20
10
0
PERCENT DEAD CELLS
0
10
20
30
40
50
TIME, HOURS
LOG CELL IN SERUM
LOG CELLS, -SERUM
LOG CELLS IN SERUM +CH
LOG CELLS, -SERUM +CH

FIG. 6



80
60
40
20
0
PERCENT DEAD CELLS
0
2
4
6
8
10
12
TIME, DAYS
+CYCLOHEXIMIDE
SERUM FREE
MEDIUM

FIG. 7

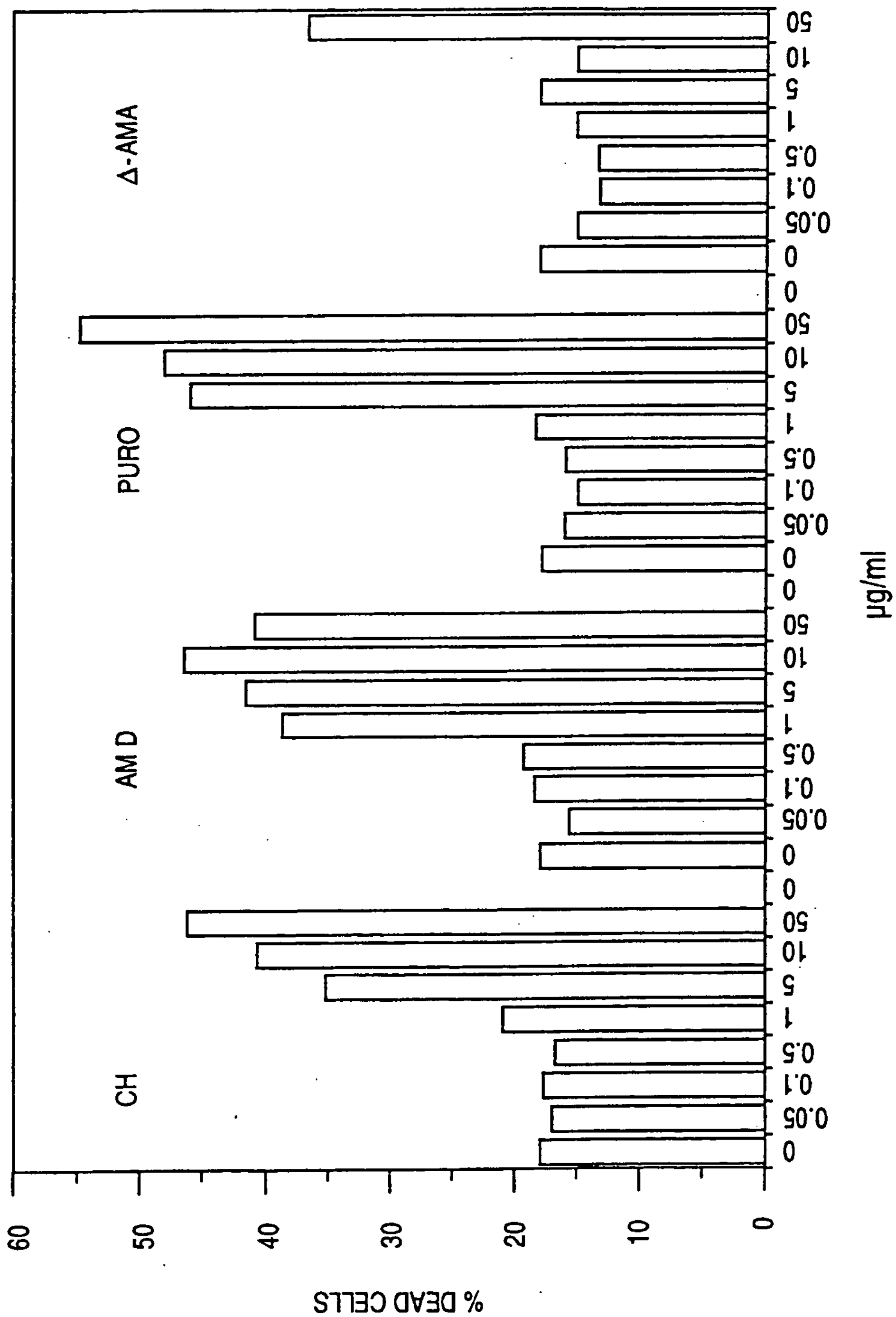

CH
AM D
PURO
Δ-AMA
% DEAD CELLS
0
10
20
30
40
50
60
0 0.05 0.1 0.5 1 5 10 50
µg/ml

FIG. 8

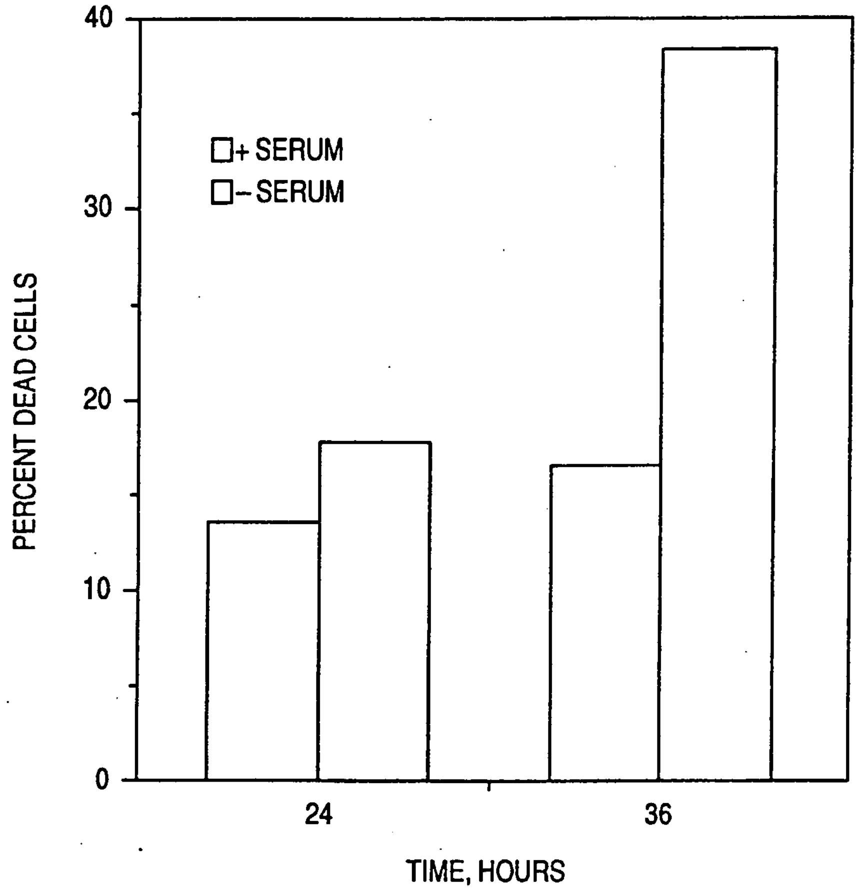


FIG. 9

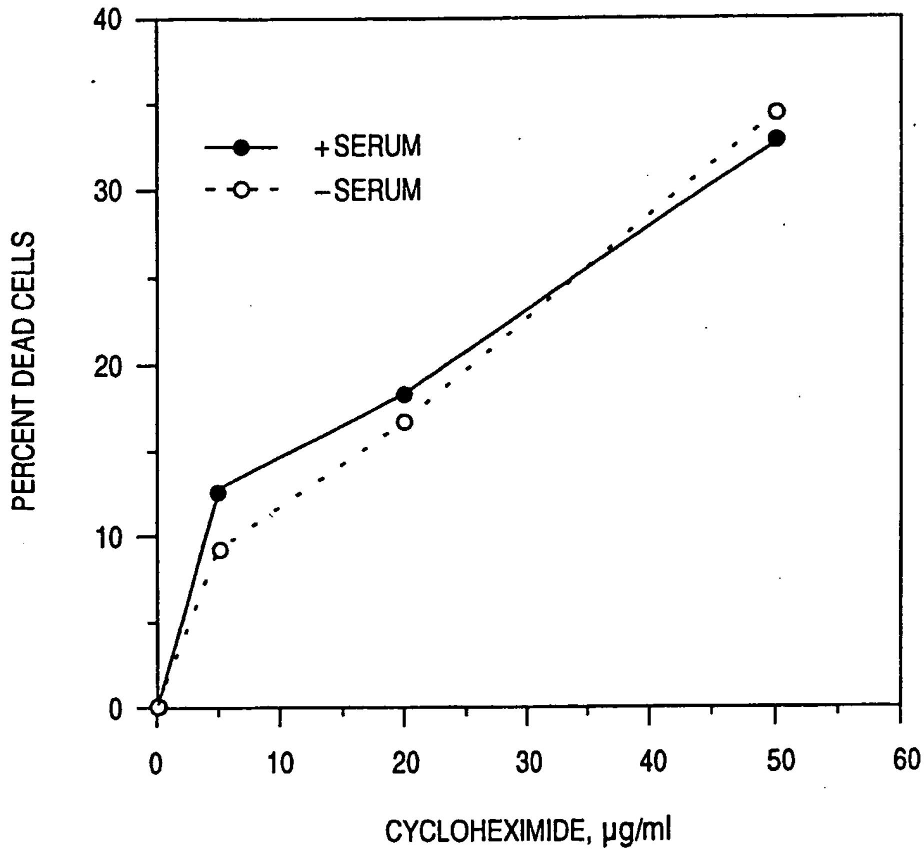

40
30
20
10
0
PERCENT DEAD CELLS
+SERUM
−SERUM
0
10
20
30
40
50
60
CYCLOHEXIMIDE, µg/ml

FIG. 10

INTERNATIONAL SEARCH REPORT

Inte. ıonal application No.
PCT/US94/04942

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12Q 1/04; C12N 5/06
US CL : 435/34, 240.23
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 29, 30, 32,34, 240.1, 240.2, 240.21, 240.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemical and Biophysical Research Communications, Volume 118, No. 2, issued 30 January 1984, Kanter et al., "Epidermal Growth Factor and Tumor Promoters Prevent DNA Fragmentation by Different Mechanisms", pages 392-399, see especially pages 397 and 398.	1-57
Y	Biochemical and Biophysical Research Communications, Volume 155, No. 1, issued 30 August 1988, Tomei et al., "Inhibition of Radiation-Induced Apoptosis In Vitro By Tumor Promoters", pages 324-331, see entire article.	1-57

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 JUNE 1994	JUL 21 1994

Name and mailing address of the ISA/US	Authorized officer
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Cellular Physiology, Volume 148, issued 1991, Kruman et al., "Apoptosis of Murine BW 5147 Thymoma Cells Induced by Dexamethasone and gamma-Irradiation", pages 267-273, see especially pages 272 and 273.	1-57
Y	Proceedings of the National Academy of Sciences, Volume 82, issued August 1985, Yavelow et al., "Nanomolar concentrations of Bowman-Birk soybean protease inhibitor suppress x-ray-induced transformation in vitro", pages 5395-5399, see especially pages 5398 and 5399.	1-57
Y	Lymphokine Research, Volume 5, No. 4, issued 1986, Duke et al., "IL-2 Addiction: Withdrawl of Growth Factor Activates a Suicide Program in Dependent T Cells", pages 289-299, see especially page 297.	1-57
Y	Nature, Volume 284, issued 10 April 1980, A. H. Wyllie, "Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation", pages 555-556, see entire article.	1-57